

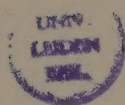
Neural and Non-neural Mechanisms in the Regulation of Bronchial Smooth Muscle Tone

in vitro evaluations

by
Jan-Anders Karlsson



LUND 1984



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To Christina and Jenny,
with love

This thesis is based mainly on the following studies, which will be referred to in the text by their Roman numerals:

- I. Influence of tracheal contraction on relaxant effects in vitro of theophylline and isoprenaline.
J.-A. Karlsson & C.G.A. Persson
Br. J. Pharmac., 74, 73-79, 1981.
- II. Effects on tracheal smooth muscle of adenosine and methylxanthines, and their interaction.
J.-A. Karlsson, G. Kjellin & C.G.A. Persson
J. Pharm. Pharmacol., 34, 788-793, 1982.
- III. Asthma mediators functionally antagonised by theophylline, enprofylline and terbutaline.
J.-A. Karlsson & C.G.A. Persson
in manuscript.
- IV. Mediators, neurotransmitters and bronchodilators in the regulation of human small airways contractility.
M.J.B. Finney, J.-A. Karlsson & C.G.A. Persson
in manuscript.
- V. Neither vasoactive intestinal peptide (VIP) nor purine derivatives may mediate non-adrenergic tracheal inhibition.
J.-A. Karlsson & C.G.A. Persson
Acta Physiol. Scand., in press.
- VI. Evidence against vasoactive intestinal polypeptide (VIP) as a dilator and in favour of substance P as a constrictor in airway neurogenic responses.
J.-A. Karlsson & C.G.A. Persson
Br. J. Pharmac., 79, 634-636, 1983.

VII. Local anesthetics selectively inhibit non-cholinergic neural contractions in guinea-pig airways.

J.-A. Karlsson & C.G.A. Persson

Acta Physiol. Scand., 120, 469-471, 1984.

VIII. Substance P antagonists and the role of tachykinins in non-cholinergic bronchoconstriction.

J.-A. Karlsson, M.J.B. Finney, C.G.A. Persson & C.

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Life Sci., in press.

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INTRODUCTION

Pathophysiology of asthma

Subjects with asthma experience periods of wheezy breathlessness between periods of relative or complete freedom from symptoms. These features have been known since antiquity and the asthmatic was vividly described already in the second century A.D. by Aretaeus (see Rosenblatt 1976). With the work of Laennec, in the mid 19th century, the knowledge of pulmonary diseases was advanced considerably, and, according to Rosenblatt (1976), he was the first to suggest the involvement of muscle fibres in bronchospasm. Numerous studies have since that time been published of the various aspects of the pathophysiology of asthma. The diversity and complexity of this disease is easily recognized when considering that, despite many attempts, a formal definition of asthma has still not been generally agreed upon (Scadding 1976 and 1983, Fletcher & Pride 1984).

Features characteristic of asthma are reversible airway obstruction correlating with variations in resistance to flow in intrapulmonary airways, unspecific airway hyper-reactivity and airway inflammation (see Scadding 1976, Boushey et al 1980). In some asthmatics these features can be related to an immediate hypersensitivity reaction (Scadding 1983). Pathological findings that may be related to the increase in resistance to flow in the airways include mucus plugging of the airways, inflammatory cell infiltrate, mucosal edema, hypertrophy of the bronchial muscle and narrowing of the airways (Thurlbeck et al 1970, Dunnill 1975, Hayes 1976, Lopez-Vidriero & Reid 1983).

The major site of airflow obstruction in chronic obstructive lung diseases is believed to be localized in the



small airways (Despas et al 1972, McFadden et al 1977), particularly during early pathological changes (Hogg et al 1968, Hogg & Cosio 1979). Also in asthma obstruction to airflow may be located in small airways. Thus, the potential importance of small airways function in lung disease has made it of particular interest to study small airways reactivity.

The lung parenchymal strip (Lulich et al 1976) is a widely used preparation in the study of small airways contractility. However, in addition to airway smooth muscle (comprising about 5% of the total volume, Bertram et al 1983, Finney et al 1984), the lung strip contains vascular smooth muscle, interstitial cells (Kapanci et al 1974) and alveolar duct smooth muscle (Miller 1921) with contractile properties. Vascular smooth muscle has been shown to be able to contribute to pharmacological responses (Mirbahar & Eyre 1980, Goldie et al 1984a) indicating difficulties in the interpretation of results obtained with the lung parenchymal strip. In the present investigation a technique described by Persson & Ekman (1976) was further developed to obtain a human bronchiolar preparation. Effects on tone of asthma mediators and bronchodilator drugs have been examined in this human small airway preparation (IV).

It has been suggested that large and small airways narrowing in asthma may be due to rapid and reversible changes in the contractile state of the smooth muscle (cf Paterson et al 1979, McFadden & Ingram 1979). The constriction is probably due to released asthma mediators of neural and non-neural origin (e.g. Lewis & Austen 1981, Samuelsson 1982, Wasserman 1983). The view that bronchoconstriction is of importance is supported by the observation that the asthmatic attack in most cases can be

immediately reversed by bronchodilator drugs (Hayes 1976, Paterson et al 1979). Effects of established and possible asthma mediators have been examined in guinea-pig (II, III, VI, VIII) and human (IV) airway preparations in vitro.

Bronchodilator drugs in asthma therapy

Asthma pharmacotherapy presently includes bronchodilator drugs like xanthines, β -adrenoceptor stimulants and anti-muscarinic agents (Paterson et al 1979), the anti-allergic agent cromoglycate (Godfrey 1983) and corticosteroids (Clark & McAllister 1983). The types of bronchodilator drugs currently in use have, surprisingly enough, been known for centuries. In 1859 Salter drew the attention to strong coffee and mental excitement as effective remedies for asthma (Persson 1980) describing the clinical efficacy of agents that we now recognize as the methyl-xanthines and sympathomimetics.

During this century the lung selectivity of the sympathomimetic agents has continuously improved. The sympathomimetics now employed (e.g. terbutaline, Persson & Olsson 1970) have, for example, significantly smaller cardiovascular actions than those previously in use (e.g. adrenaline, isoprenaline; see Tattersfield 1983). It was not until recently the synthesis of the first comparatively lung-selective xanthine derivative, enprofylline (3-propylxanthine), was reported (Persson & Kjellin 1981, Persson et al 1981). Enprofylline is about 5 times more potent than theophylline as a bronchodilator in animals and man (for ref. see Persson 1984) but seems to lack many of theophylline's extrapulmonary actions, e.g. CNS-stimulant and diuretic actions (see Persson 1984, and below).

In studies of bronchodilator drug actions, attention seems to have focused mainly on β -adrenoceptor agonists. Thus, comparatively little appears to be known about airway relaxant characteristics of xanthine derivatives. In the present investigation various aspects on relaxant properties of xanthines and β -adrenoceptor stimulants, such as efficacy, species- and age-dependent effects and tolerance development, have been examined in isolated airway preparations obtained from animals and man (I-IV, and below).

β -adrenoceptor agonists are generally thought to induce smooth muscle relaxation, by stimulation of adenylate cyclase and thereby increasing the intracellular concentration of cyclic adenosine 3' 5'-monophosphate (cAMP) (see Andersson 1973). Relaxant actions of xanthines have also been proposed to be mediated by cAMP, but instead via an inhibition of cAMP-degrading phosphodiesterase (PDE) activity (e.g. Butcher & Sutherland 1962, Sutherland et al 1968). Several other mechanisms of xanthine-induced relaxatory actions have been considered, including antagonism at receptors for the purine nucleoside adenosine (see Rall 1980, Persson 1984). Some aspects on the mechanism behind the airway smooth muscle relaxant actions of xanthines have been studied in the present investigation (I-IV).

Neurogenic regulation of airways tone, prospects for bronchodilator drugs?

The airway hyperreactivity in asthma has been considered to be due to a dysfunction in the autonomic regulation of airway smooth muscle tone (see Boushey et al 1980). Bronchoconstriction may arise either from an enhanced activity of constrictive nerves or from an impaired activity of inhibitory nerves. Many asthmatics seem to

have an exaggerated activity of bronchoconstrictive cholinergic nerves since in these subjects atropine-like drugs (by blocking the muscarinic effects of acetylcholine released from cholinergic nerves; Dixon 1906, Loewi 1921) produce bronchodilation (Simonsson 1977, Gross & Skorodin 1984). The enhanced vagal efferent activity may depend on the activation of a vagal reflex pathway (see Boushey et al 1980, Widdicombe 1981, Gross & Skorodin 1984) or on an increased activity of post-junctional muscarinic receptor sites (see Boushey 1984). Thus, the study of cholinergic mechanisms seems to be of importance for the understanding of neural regulation of airways calibre. Muscarinic receptor-mediated effects on tone have been studied in guinea-pig (I, III, V-VII) and human (IV) airway preparations.

The presence of non-cholinergic non-adrenergic contraction-mediating nerves has been demonstrated in the guinea-pig hilus bronchi (Grundström et al 1981), and in the intact guinea-pig, electrical stimulation of the vagus nerve produced an atropine-resistant bronchoconstriction (Lundberg et al 1983c). A non-cholinergic bronchoconstrictive nervous system has been suggested to be present also in human airways (Lundberg et al 1983b).

Histochemical studies have demonstrated immunoreactivity to the bronchoconstrictive tachykinin peptide substance P(SP) (Andersson & Persson 1977, Nilsson et al 1977) in nervous structures, possibly sensory neurons, within the airways (Nilsson et al 1977, Lundberg et al 1983a, 1984). Capsaicin, which depletes sensory neurons of mediators (e.g. SP) and causes the nerve fibres to degenerate (Jancso et al 1977, Jessell et al 1978), was found to reduce immunoreactivity to SP in the guinea-pig lung and to abolish the non-cholinergic non-adrenergic bronchocon-

striction (Lundberg et al 1983a,c, 1984). Furthermore, capsaicin has been shown to be a bronchoconstrictor in vitro and in vivo in animals and man (e.g. Lundberg et al 1983b,c, Fuller et al 1984). Partially based on these studies, Lundberg and co-workers considered SP a possible mediator of non-cholinergic bronchoconstriction.

The synthesis of SP-analogues with SP antagonistic properties has recently been reported (e.g. Rosell & Folkers 1982, Leander et al 1983, Regoli et al 1984). In the present investigation, the possible involvement of SP in the non-cholinergic non-adrenergic contraction of the guinea-pig hilus bronchi was examined by use of SP antagonists (VI, VIII). In addition, some aspects on the specificity of the SP antagonists were evaluated (VIII).

Neurogenic inhibition of human airways has been demonstrated to be mediated by non-cholinergic non-adrenergic nerves (Richardson & Beland 1976, Davis et al 1982, Taylor et al 1984). This inhibitory nervous system appears to present many similarities to that in the gastro-intestinal tract (Richardson & Bouchard 1975). A defect in these nerves is believed to be the origin of a spasm of the intestinal muscle (leading to Hirschsprung's disease), and Richardson & Bouchard (1975) have suggested that perhaps a similar dysfunction could explain the airway hyperreactivity in asthma and chronic bronchitis. Partially based on work with the guinea-pig isolated trachea, in which the predominant inhibition also is mediated by non-adrenergic nerves (Coburn & Tomita 1973), the non-adrenergic neurotransmitter has been considered to be a purine derivative (Coleman & Levy 1974, Souhrada et al 1980, Satchell 1982) or vasoactive intestinal peptide (VIP, Matsuzaki et al 1980). The features of this nervous system and the possibility that the non-adrenergic inhibition in the guinea-

pig trachea is mediated by any of these agents have been examined in studies V and VI.

In vitro methodology in the study of airway smooth muscle reactivity

The present investigation has restricted itself to the use of in vitro methods in the study of bronchodilator drug actions and mechanisms in the regulation of airways tone. Results obtained in isolated smooth muscle preparations should be cautiously extrapolated to the in vivo situation because little is known about actual concentrations of asthma mediators, of contributory drug actions on non-smooth muscle cells, and because in isolated tissues hormonal, neural and other modulatory endogenous mechanisms are largely lacking. However, in vitro techniques are particularly suitable for quantitative evaluations and reactivity of airway smooth muscle from defined parts of the bronchial tree from animals and man can be examined.

AIMS

The present investigation was conducted in vitro to study some selected aspects of drug and mediator actions and neural mechanisms of possible relevance for bronchodilator therapy.

The specific aims of this investigation can be summarized:

to examine the relaxant effects of xanthines and β -adrenoceptor stimulants in guinea-pig and human airway smooth muscle preparations

to determine which positions for alkyl substitution in the xanthine molecule that are most important for bronchodilator properties

to study some aspects of the mechanism of the xanthine-induced relaxation of airway smooth muscle

to study the constrictive effects of possible asthma mediators in guinea-pig and human airway smooth muscle preparations

to characterize electrically induced neurogenic responses in guinea-pig isolated airway preparations

to examine the possibility that the non-adrenergic neurogenic inhibition in the guinea-pig trachea is mediated by a purine derivative or VIP

to attempt to identify the mediator of the non-cholinergic contraction in the guinea-pig hilus bronchi by use of SP analogues with SP-antagonistic properties and to evaluate the specificity of the SP antagonists.

METHODS

Isolated airway preparations

Guinea-pig tracheal preparation (I-III, V-VII)

Tracheas were excised from guinea-pigs of either sex. A tracheal ring was obtained by cutting out two adjoining cartilage rings. In some experiments the ring was opened by cutting through the cartilage. The open ring was mounted vertically by use of cotton threads and the intact ring, when employed, on two stainless steel prongs in organ baths containing temperature-controlled (37°C) and gassed (5% CO_2 in oxygen; carbogen) Krebs solution.

Guinea-pig bronchial preparation (VI-VIII)

The lung together with the thoracic part of the trachea was resected from guinea-pigs of either sex. Tubal segments (about 2-3 mm long) were taken from the hilus bronchi of the left and right caudal lobes and mounted on stainless steel prongs in organ baths containing heated (37°C) and carbogenized Krebs solution.

Human bronchiolar preparation (IV)

Human lung tissue was obtained at lobectomy or pneumonectomy performed for carcinoma of the lung. Macroscopically normal tissue specimens were used. A flexible plastic tube (outer diameter 0.6 mm) was gently inserted as far as possible into the lumen of a bronchus. Tubal segments of bronchioles (about 2 mm long, diam. 0.6-1.5 mm) were prepared by dissecting them free of surrounding tissue (the tubing ensuring correct identification). The bronchioles were mounted on metal prongs in organ baths as

described above. The airways nature of the preparations was verified by a relaxant response to noradrenaline (10^{-7} - 10^{-5} M) and by histology.

Recording of tension in airway preparations

Isometric tension was measured by use of Grass force-displacement transducers (FTO3) and recorded on a Grass polygraph model 5 or 7.

Electrical field stimulation of guinea-pig airway preparations (V-VIII)

Guinea-pig airway preparations were electrically stimulated by use of a Grass S48 or S88 stimulator and Grass SIU5 units. Tracheas and bronchi were transmurally stimulated at a supramaximal or maximum voltage, respectively. With a pulse duration of ≤ 0.6 ms, tetrodotoxin-sensitive responses were obtained. Frequency and duration of stimulation varied in the different experiments.

Frog isolated sciatic nerve preparation (VIII)

Both sciatic nerves were isolated from *Rana pipiens*. The nerves were desheathed, split in halves longitudinally and placed in nerve baths containing MOPS solution at room temperature (20° C). They were continuously electrically stimulated (Grass S48 stimulator) at supramaximal voltage (frequency 1 Hz, pulse duration 0.05 ms) and the compound action potential (APc) was recorded by use of the sucrose gap technique (Stämpfli 1954). APcs were recorded with a Neurolog device and displayed on a Tektronix oscilloscope that was on line with a Hewlett-Packard computer.

Drugs and statistics

Drugs were added as injections to the organ bath either as a single dose or in a cumulative progression of concentrations. Control experiments were always performed with the solvents used. Geometric mean $\pm$ SEM values were calculated for each concentration of the substances used. When evaluating adenosine antagonism of xanthines, pA_2 -values and slopes were calculated according to Arunlakshana & Schild (1959). Statistical analyses were made by use of the Student's t-test for unpaired observations or by a one-way analysis of variance.

RESULTS AND DISCUSSION

Some comments on the airway preparations used

An in vitro airway smooth muscle preparation is an important tool in the study of airway reactivity and the mode of action of bronchoactive agents. The present investigation has utilized guinea-pig intact or open (Persson, H. & Sonmark, B., unpublished method) tracheal rings and tubal segments from the hilus bronchi of the caudal lobes. The tracheal ring preparation had the advantage of being much easier to prepare than the commonly used tracheal chains (Castillo & De Beer 1947, Akcasu 1952, Foster 1960) and spirals (Patterson 1958, Constantine 1965) and the ring preparation seemed to be at least as sensitive to mediators and drugs as the more elaborate tracheal in vitro preparations.

An interesting feature of the guinea-pig trachea is the high spontaneous contraction that it develops in vitro. The contraction does not seem to be neurogenically mediated; tetrodotoxin is without effect on tone (Karlsson J.-A., unpublished observation). Neither muscarinic receptor (Carlyle 1963) nor α -adrenoceptor (Foster 1966) activation seems to contribute. Of particular interest in relation to the mechanism behind the smooth muscle relaxant action of xanthines (see below), are the observations that addition of dipyridamole (in concentrations blocking the cellular uptake of the purine nucleoside adenosine, Kolassa et al 1970) or of adenosine deaminase (Coleman 1976, II, Karlsson J.-A. & Persson C.G.A., unpublished observations) does not affect the tone. These results indicate that endogenous adenosine is not involved in the maintenance of the intrinsic tone. Indomethacin, on the other hand, relaxes spontaneously contracted tracheas

and the tone is, therefore, suggested to be mediated by cyclooxygenase products (Farmer et al 1974, Orehek et al 1975). However, inhibitors of the arachidonate lipoxygenase products also reduce tracheal tone (e.g. Mitchell & Denborough 1980, Lundblad K.A.L., Karlsson J.-A. & Persson C.G.A., unpublished observations), why its cause remains conjectural.

In human isolated bronchioles, immediate and reversible tension changes were produced by constrictive and relaxatory agents. These results are compatible with the view that a reduction in small airways calibre in lung disease may in part be due to rapid changes in the contractile state of the smooth muscle (McFadden & Ingram 1979). In contrast to the lung parenchymal strip preparation, these bronchioles should indeed reflect small airways reactivity.

Effects on airways tone of established and possible non-neural asthma mediators

The bronchoconstriction in asthma is likely to be due to released mediators of neural and non-neural origin (for ref. see Introduction). The lung mast cell is generally considered the major source of non-neurally derived constrictive and pro-inflammatory mediators although other cell types like the basophils, macrophages and platelets also have the potential to release this material (e.g. Lagunoff 1983, Morley 1983, Wasserman 1983). In addition, these cells contain chemotactic factors, enzymes and other agents that may contribute to the pathological changes in asthma (e.g. Wasserman 1983, Page et al 1984).

Histamine is a major constituent of mast cells and is since long known as a bronchoconstrictor in animals and

man. Histamine was in the present investigation employed as a standard bronchoconstrictor. Although it has a low potency, histamine, together with carbachol, produced the largest degrees of airway contraction of the mediators studied (III, IV).

Prostaglandin D_2 (PGD_2) is the quantitatively predominant arachidonate cyclooxygenase product released from sensitized human lung tissue (probably from mast cells) after challenge in vitro with antigen (Schulman et al 1981). PGD_2 was in this investigation found to contract human bronchioles with a potency and an efficacy similar to histamine's (IV). PGD_2 has recently been shown to be a bronchoconstrictor in man (Hardy et al 1984) and may be as important as histamine to small airways constriction in obstructive lung diseases.

Antigen produces a slowly developing contraction in guinea-pig tracheas which may be due to the release of SRS-A (e.g., Forsberg & Sörenby 1979, Andersson 1982). SRS-A has been identified as a mixture of the leukotrienes C_4 , D_4 and E_4 (Murphy et al 1979, Lewis et al 1980, Morris et al 1980) which by themselves contract airway smooth muscle (e.g. Dahlén et al 1980, Hanna et al 1981). In the present investigation the leukotrienes C_4 and D_4 were found to be almost one order of magnitude more potent than carbachol but to produce a slightly smaller degree of contraction in guinea-pig tracheas (III) and human bronchioles (IV, Karlsson J.-A. unpublished observations). The features of the contractions induced by leukotrienes were similar to those induced by antigen, but still it cannot be excluded that other as yet unknown mediators contributed to the antigen-induced response (Burka 1982, Woodward et al 1983, Lamm et al 1984).

The purine nucleoside adenosine has been proposed to be an asthma mediator (Fredholm et al 1979, Cushley et al 1983) and Cushley et al (1983) showed that inhalation of large doses of adenosine produced bronchoconstriction in asthmatics. Bronchoconstriction was also induced by this nucleoside in guinea-pig isolated perfused lungs (Kröll et al 1984). However, adenosine has been shown to be practically without constrictive properties in guinea-pig isolated tracheas with a spontaneous tone (e.g. Coleman 1976, Farmer & Farrar 1976). These findings have been confirmed (II) and extended in the present investigation by the observation that only relaxant responses were produced in tracheas with larger degrees of contraction (V). In human isolated bronchi (Davis et al 1982, Finney et al 1983) and bronchioles (IV) adenosine was either without effect or produced a weak contraction. The lack of correlation between in vivo and in vitro effects of adenosine indicates that the bronchoconstriction in vivo may not be due to a direct action on the smooth muscle.

Although the amount released and the relevant concentration at target cells are unknown, many of the mediators found to contract human small airways may be of importance in asthma. The contractile characteristics of the asthma mediators studied differed greatly (II, III, IV), suggesting that different cellular mechanisms were activated. However, apart from specific receptor activation, little is known about the biochemical pathways involved in the contractile responses.

Relaxant characteristics of xanthines and β -adrenoceptor-stimulants

Bronchodilator responses differ little with species but depend on airway contraction

Relaxant effects of methylxanthines and β -adrenoceptor stimulants were examined in guinea-pig, rat and human airway preparations. Only guinea-pig and human airway preparations gained a spontaneous tone and, therefore, bronchodilators were without effect in resting rat tracheas (Jamieson 1962, Lulich & Paterson 1980; Fig. 2). In some contrast with earlier work (e.g. Åberg et al 1973) the sensitivity of the airway preparations to xanthines and β -adrenoceptors was found to vary little between species (I, III, IV, cf. Fig. 2). With a moderate degree of contraction, in guinea-pig tracheas and human bronchioles, a significant relaxation was produced by xanthines and β -adrenoceptor stimulants already at clinically relevant concentrations (I, IV). These data are in agreement with the view that bronchodilator responses in the guinea-pig trachea show many similarities to those in human bronchi (Hawkins & Schild 1951).

A comparison between relaxant properties of xanthines and β -adrenoceptor stimulants can be made by varying the degree of airway's contraction. It is well known that the effect of a β -adrenoceptor agonist is diminished by an increase in the tone (van den Brink 1973, Jones et al 1974, Buckner & Saini 1975), but the effects of xanthines during these conditions have been less well documented. In the presence of large concentrations of carbachol, the relaxant effects of both theophylline and isoprenaline were now found to be reduced (I). At a maximum carbachol contraction, the response to isoprenaline, amounted only

to about one half of that of theophylline, (I). Concomitantly, the potency ratio between the drugs was reduced about tenfold (I). Similar observations have been made in human isolated bronchi (Karlsson J.-A. & Persson C.G.A., unpublished observations). These results show that xanthines are more effective relaxants of highly contracted airway smooth muscle than β -adrenoceptor agonists. The importance of this difference in vivo has not been assessed.

Methylxanthines and β -adrenoceptor stimulants are generally considered potent relaxants of both central and peripheral mammalian airways (e.g. Hawkins & Schild 1951, Persson & Ekman 1976). It was thus surprising when Davis et al (1980) reported that large concentrations (10^{-7} - 10^{-3} M) of the β_2 -adrenoceptor agonist salbutamol were needed to relax human bronchi with an intrinsic tone and that this drug was virtually without effect in histamine-contracted preparations. In the present investigation terbutaline was found to be a potent relaxant of carbachol-contracted human bronchioles (IV). Four out of eleven bronchioles were completely relaxed by terbutaline suggesting that β_2 -adrenoceptor stimulants are potent relaxants of peripheral airways, at least in some human subjects (IV). The xanthine derivatives theophylline and enprofylline, consistently relaxed the human bronchioles (also those found to be unresponsive to terbutaline) (IV). Thus, xanthines and a β_2 -adrenoceptor agonist, although less frequently, were found to relax a human small airway preparation. It is conceivable that a relaxation of small airways relaxant action contributes to the anti-asthmatic efficacy of these drugs.

Structure-activity relationships for xanthines

With xanthine and 15 methylxanthines it was quantitatively confirmed that the 3- and then the 1-position would be most important for improving the guinea-pig tracheal relaxatory potency (Fig 1, II; cf Persson C.G.A., Ekman M., Erjefält I. & Kjellin G., in the files of AB Draco). Unsubstituted and 9-methylated xanthines were weakly active (Fig 1, II). An increase in the size of the alkyl group in the 3-position enhances the potency considerably, thus 3-propylxanthine is about 15 times more potent than 3-methylxanthine (cf Fig 1, Persson et al 1982b). The findings with the methyl substituted xanthines generally agree with observations made with xanthine derivatives containing larger alkylsubstituents (Armitage et al 1961, Persson C.G.A., Ekman M., Erjefält I., Kjellin G., in the files of AB Draco). It is conceivable that the structure-activity relationships obtained in vitro also are valid for bronchodilation in vivo, since enprofylline (cf. Fig. 1, III; Lunell 1983, Persson 1984), but not caffeine (Becker et al 1984), seems to be more potent than theophylline as a bronchodilator in animals and man.

On tolerance development to xanthines

Prolonged exposure of airway smooth muscle to high concentrations of a relaxant drug may induce tolerance, i.e. a decreased responsiveness to subsequent exposure to the drug and perhaps to other relaxants as well. Tolerance may develop in the human lung after repeated exposure to high doses of β -adrenoceptor stimulants (e.g. Jenne 1979, Paterson et al 1979). It can readily be induced in animal lung tissue and in human bronchi in vitro (e.g. Douglas et al 1977, Lin et al 1977, Avner & Noland 1978, Davis & Conolly 1980, Holmberg et al 1981). It appears that the

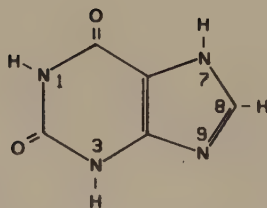
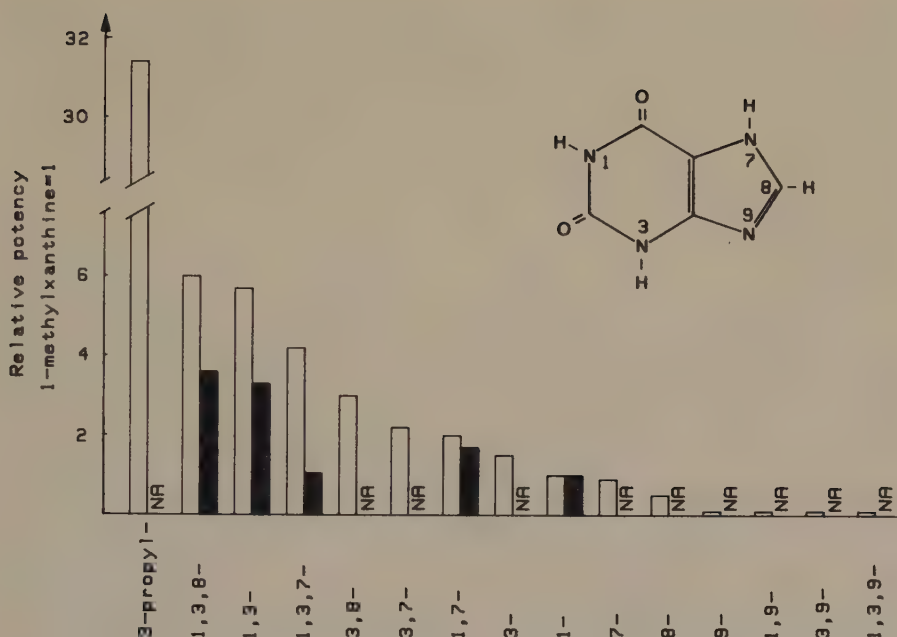


FIGURE 1

Effects on the isolated guinea-pig trachea by 3-propylxanthine (enprofylline) and different methylxanthines. The positions for methylsubstitution of the xanthine molecule are indicated (see also insert). The open bars illustrate tracheal relaxant potencies based on EC50 values of the xanthines ($n=4$) compared with that of 1-methylxanthine (EC_{50} , $mean \pm SEM = 1.81 \pm 0.07$ mM, $n=4$; potency=1). At EC_{50} , $SEM < 15\%$ for all compounds. The filled bars illustrate adenosine antagonism. Only 5 of the tested xanthines exhibited consistent adenosine antagonism. Schild plots had slopes that did not differ from 1, the theoretical value for competitive antagonism. Relative potencies were obtained by comparing the pa_2 values with that of 1-methylxanthine ($pa_2=4.40$: potency=1). NA = compounds not active as adenosine antagonists.

development of tolerance is related to prostaglandin synthesis, since it has been shown to be prevented by pretreatment with indomethacin (e.g. Douglas et al 1977). The validity for the clinical situation of observations on tolerance in isolated airway preparations remains obscure (Bruynzeel 1984).

Data on tolerance development to theophylline are scarce but this drug is frequently considered incapable of inducing tolerance in airway smooth muscle (Avner & Noland 1978, Jenne 1979, Paterson et al 1979). Contradictory to this view, Benoy et al (1975) reported that the sensitivity to theophylline was reduced in guinea-pig lungs previously exposed to moderate doses of the xanthine. In view of this finding it was of interest to examine the sensitivity of airway smooth muscle to enprofylline and isoprenaline after long-term treatment with this xanthine derivative. The responsiveness to both enprofylline and isoprenaline was found to be unchanged in tracheas obtained from rats fed food-pellets containing enprofylline for 2 weeks or 6 months (Fig 2, Karlsson & Persson 1982), the latter period being a considerable portion of the life span of the rat. The daily dose was well above the "therapeutic" dose in man (Lunell et al 1982), and the high bioavailability of enprofylline in the rat, ($78.1 \pm 3.0\%$, $n=16$, of administered enprofylline recovered intact in rat urine, calculated from 24 hours urine samples; see Persson et al 1982a) indicated that the airways had been exposed to enprofylline. It is conceivable that the in vitro findings also showed what would happen in vivo at the level of airway smooth muscle. In a similar way, the decreased responsiveness after administration of β -adrenoceptor stimulants to intact animals has been demonstrated both in vitro and in vivo (e.g. Douglas et al 1977, Holmberg et al 1981) It is suggested that xanthines may

have a negligible ability to induce tolerance to themselves or to other relaxants in airway smooth muscle.

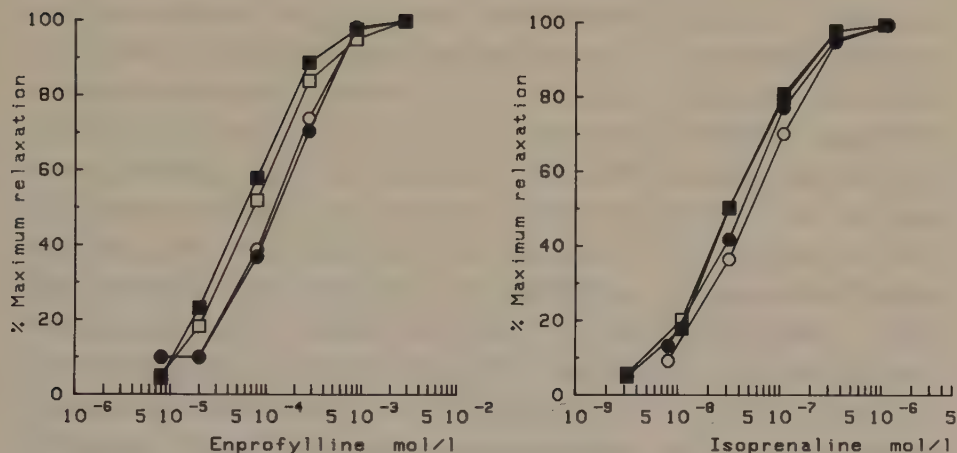


FIGURE 2

Relaxant concentration-response curves to enprofylline and isoprenaline in rat isolated tracheal preparations contracted by $0.44 \mu\text{M}$ carbachol. Tracheas were obtained from rats fed ordinary food-pellets for 2 weeks ($\square$) or 6 months ($\circ$) and from rats fed food-pellets containing enprofylline for 2 weeks ($\blacksquare$) or 6 months ($\bullet$). The mean daily doses of enprofylline were $492 \mu\text{mol/kg}$ and $606 \mu\text{mol/kg}$ during the 2-week and 6-month periods, respectively. On the ordinate relaxation is expressed as per cent of its own maximum relaxation. Each curve is the mean of 7 to 14 animals. $\text{SEM} \leq 7\%$ in each point.

On age-dependent relaxant effects of xanthines

Bronchodilator actions of β -adrenoceptor stimulants seem to be affected by age. Isoprenaline has been shown to be less potent in airway preparations from old than from young animals (Åberg et al 1973, Brink et al 1980). It is not known if bronchodilator actions of xanthines are likewise affected by age. Therefore, relaxant effects of enprofylline were compared with those of isoprenaline in tracheas obtained from rats of different age. In accordance with previous findings in the guinea-pig trachea, the potency of isoprenaline was reduced in tracheas from old rats (Table 1). In contrast, both potency and efficacy of enprofylline were unaffected by age (Table 1). These data would therefore suggest that xanthines, as opposed to β -adrenoceptor stimulants, do not possess age-dependent relaxant actions in animals, further supporting the view of a difference in the mechanism by which these two types of drugs induce airway smooth muscle relaxation.

TABLE 1

Relaxant effects of enprofylline and isoprenaline in isolated tracheas obtained from young (1.5 months) and old (7 months) rats. Negative log molar EC50 ($\pm$ SEM) values from n animals are shown.

		Young	n	Old	n	
Enprofylline	-log EC50	3.92 $\pm$ 0.08	9	3.92 $\pm$ 0.05	12	NS ^b
	max relaxation ^a	91.2 $\pm$ 5.2	9	78.9 $\pm$ 3.0	12	NS
Isoprenaline	-log EC50	7.87 $\pm$ 0.06	6	7.29 $\pm$ 0.07	12	P<0.001
	max relaxation	42.9 $\pm$ 3.3	6	50.9 $\pm$ 5.2	12	NS

^a % inhibition of the carbachol (0.44 μ M)-induced contraction

^b NS = not significant, Student's t-test

Functional antagonism to asthma mediators

It is generally believed that xanthines and β -adrenoceptor agonists are effective as relaxants irrespective of which asthma mediator has constricted the airway (e.g. Skidmore 1982) although supportive data seem to be scarce (Olsson & Persson 1976). Rather, several studies have indicated that the potency and efficacy of bronchodilator drugs may vary with constrictor. For example, Spilker & Minatoya (1975) found isoprenaline to be almost two orders of magnitude less potent in potassium-depolarized than in histamine-contracted guinea-pig tracheas, and Mitchell & Denborough (1979) found the relaxant effects of isoprenaline and theophylline to be different in histamine- and acetylcholine-contracted feline tracheas, respectively. The possibility that xanthines and β -adrenoceptor stimulants selectively antagonize a single mediator was examined in the guinea-pig isolated trachea (III). In essence, these results showed that theophylline, enprofylline and terbutaline had a similar potency irrespective of whether antigen or anyone of several possible asthma mediators had contracted the trachea (III), i.e. they exerted a functional antagonism (Offermeier & van den Brink 1974, Olsson & Persson 1976). Airways narrowing in asthma is believed to be caused by a large number of released asthma mediators acting in conjunction on smooth muscle (for ref. see Introduction) and it is possible that xanthines and β -adrenoceptor agonists are effective in asthma because they act as functional antagonists to the mediators.

If this assumption is valid, these types of drugs, rather than agents that specifically antagonize one mediator only, would be expected to be effective in reversible obstructive lung diseases.

On the mechanism of xanthine-induced relaxation of airway smooth muscle

Cytoplasmic Ca^{2+} is vital for the contractile mechanism in smooth muscle since it activates the contractile proteins (actin and myosin) via a series of protein phosphorylation reactions (see Ebashi 1980). An increased availability of Ca^{2+} to the contractile proteins thus induces a contraction of the muscle, whereas a withdrawal of Ca^{2+} from the vicinity of the contractile machinery induces a relaxation. Also in airway smooth muscle Ca^{2+} seems important for contractility (e.g. Lanerolle & Stull 1980, Sparrow et al 1984). Several mechanisms for the smooth muscle relaxatory effect of theophylline have been proposed. At the cellular level, theophylline has been shown to inhibit cAMP-PDE activity (Sutherland & Rall 1958, Butcher & Sutherland 1962), and to affect translocation of intracellular Ca^{2+} (Syson & Huddart 1976). Furthermore, methylxanthines have been shown to enhance the effects of catecholamines (Kalsner 1971, Kalsner et al 1975, Highbee et al 1982), to attenuate prostaglandin-mediated responses (Vinegar et al 1976, Horrobin et al 1977) and to antagonize competitively the effects of adenosine (for ref. see below). These mechanisms have been considered of importance for bronchodilation, although inhibition of PDE activity and antagonism to endogenous adenosine have attracted most attention.

Inhibition of phosphodiesterase activity

cAMP, the presumed second messenger of β -adrenoceptor induced smooth muscle relaxation (Andersson 1973), is metabolized to 5'AMP by intracellular PDE activity (Sutherland & Rall 1958, Butcher & Sutherland 1962). The observation that methylxanthines inhibit cAMP-PDE activity

was taken as strong evidence for the importance of this mechanism in methylxanthine-induced bronchodilation (e.g. Butcher & Sutherland 1962, Sutherland et al 1968). Further support for this view stems from the finding that a good correlation existed between inhibition of PDE activity in vitro and bronchodilation (Triner et al 1977, Polson et al 1978, Newman et al 1978).

Sutherland et al (1968) hypothesized that a drug that stimulates cAMP formation should be potentiated by an inhibitor of PDE activity. Indeed, caffeine and adrenaline were found to synergistically increase the cAMP level in rat lung tissue (Sutherland et al 1968). Synergism between theophylline and β -adrenoceptor stimulants has occasionally been shown in the relaxation of airway smooth muscle (Bertelli et al 1973, Mitchell et al 1979) although most studies have found only an additive interaction (see Hanna & Roth 1979). In the present study, combinations of maximally effective concentrations of theophylline and isoprenaline did not produce a larger relaxation than theophylline alone (I). Moreover, the concentration-response curve to theophylline was almost identical whether obtained in the absence or presence of a maximally effective isoprenaline concentration (I). These data seem difficult to reconcile with the view that inhibition of PDE activity is important for the relaxant effects of xanthines. Furthermore, theophylline has been shown to be able to relax isolated airway preparations apparently without affecting the cellular concentration of cAMP (e.g. Lohmann et al 1977, Kolbeck et al 1979). At maximally effective therapeutic concentrations theophylline is only a weak inhibitor of PDE activity (Beavo et al 1970, Kolbeck et al 1979, Bergstrand 1980). Further support for the view that inhibition of PDE activity is unimportant for bronchodilation emanates from the findings that highly

selective and potent PDE inhibitors failed to produce bronchodilation in man (see Persson 1984).

Adenosine antagonism

Adenosine has been suggested to play a role in many physiological processes (see Baer & Drummond 1979, Fredholm & Hedqvist 1980). Based on biochemical studies (actions on adenylate cyclase) adenosine has been proposed to act on two different types of extracellularly located receptors: A_1 -receptors mediating inhibition of adenylate cyclase activity and A_2 -receptors mediating enhancement of activity (van Calcar et al 1978). These two types of receptors have been shown to have differential sensitivity to a series of adenosine analogues (see Baer & Drummond 1979) indicating that the receptor subtype can be pharmacologically characterised. Theophylline, already at therapeutic concentrations, is generally considered a universal antagonist at cell surface receptors for adenosine. Accordingly, adenosine antagonism has been proposed to be the mechanism behind many of theophylline's pulmonary and extrapulmonary actions (see Rall 1980, Persson 1984).

The concentration of adenosine has been shown to increase during pathological conditions such as local hypoxia (Mentzer et al 1975), perhaps suggesting a role for this nucleoside in the lung.

Adenosine may contract isolated airway smooth muscle (Fredholm et al 1979, II) and produce a bronchoconstriction in asthmatics (Cushley et al 1983, 1984). This nucleoside has also been shown to enhance histamine release from cells present in the lung (Marquardt et al 1978, Fredholm 1980, Welton & Simko 1980). These effects

were all antagonized by theophylline, and partly based on these results adenosine antagonism was proposed as the mechanism for xanthines' relaxatory actions (Marquardt et al 1978, Fredholm 1980, Welton & Simko 1980, Cushley et al 1983, 1984).

In order to examine the importance of adenosine antagonism for airways relaxation, a structure-activity study with xanthine derivatives was conducted (II). Antagonism of adenosine-induced guinea-pig tracheal relaxation (mediated via A_2 -receptors, Brown & Collis 1982) was studied. Methyl substitution in the 1-position of the xanthine molecule was found to be essential for potent adenosine antagonism (Fig. 1, II), whereas both the 3- and the 1-position were important for tracheal relaxation (see above). These findings confirmed the new observation made by Persson et al (1981) that potent bronchorelaxant xanthine derivatives practically devoid of adenosine-antagonistic properties can be produced: enprofylline, which is almost devoid of adenosine-antagonistic properties (e.g. Persson et al 1981, Persson et al 1982b), is about 5 times more potent than theophylline as an anti-asthmatic drug in man (see Lunell 1983, Persson 1984). Also, dipyridamole, which enhances the amount of adenosine available to cell surface receptors, did not affect tone in isolated airway preparations (II, Davis et al 1982) nor change lung resistance to flow in man (Ruffin & Newhouse 1981). Thus, adenosine antagonism may not be important for airway relaxatory effects of xanthine derivatives (II, III, IV).

In this context, it is of interest to note that no purine or pyrimidine nucleoside or nucleotide with potent constrictive effects in airway isolated preparations has been identified (Lundblad et al 1984).

Despite the apparent lack of importance of adenosine antagonism for bronchodilation, this mechanism may be more relevant to some of theophylline's extrapulmonary actions (see Rall 1980, Andersson & Persson 1980, Persson 1984) as for example its CNS-stimulant effects: adenosine agonists have sedative effects (Barraco et al 1983) and adenosine is a depressant of nerve cell firing in many areas of the brain (Phillis et al 1979, Perkins & Stone 1980, Daly et al 1981).

A readily accessible neural tissue that pharmacologically has many similarities to the CNS, is the guinea-pig myenteric plexus-longitudinal muscle preparation (North 1980). In this preparation, adenosine, by acting as a neurodepressant (at A_1 -receptors, Paton 1981) inhibited electrically induced twitches (due to the release of acetylcholine, Sawynok & Jhamandas 1976, Karlsson & Persson 1981). As was observed in the tracheal preparations (II see above) and in human fibroblasts in vitro (in which adenosine enhances the cAMP content, Bruns 1981) 1-alkylated xanthine derivatives antagonized the adenosine-induced depression (Karlsson & Persson 1981, Persson et al 1982a). These data indicate that presently available, xanthine derivatives with adenosine-antagonistic properties do not separate between A_1 and A_2 receptors. Xanthines having adenosine-antagonistic properties in the myenteric-plexus preparation increased spontaneous locomotor activity in mice (Persson et al 1982b, Bruns et al 1983, Karlsson et al 1984), which may be an early sign of CNS-stimulation. The adenosine antagonist theophylline, but not the adenosine non-blocking drug enprofylline, was in large doses able to produce massive generalized convulsions in many species (Persson et al 1981, Persson & Erjefält 1982). These data would thus suggest that theophylline stimulates the CNS via an antagonism of the

neurodepressant action of endogenous adenosine. Examples of other extrapulmonary actions of theophylline that seem to be mediated via adenosine antagonism, and where enprofylline has been shown to be practically without effects, include diuretic effects (Osswald 1975, Persson et al 1982a), some metabolic actions (Andersson et al 1984, Johanneson et al 1984) and stimulation of the respiration (Hedner et al 1982, Lagercrantz et al 1984).

Unknown mechanism(s)

It is presently not known if xanthine-induced smooth muscle relaxation is initiated via specific cell surface structures. Theophylline and caffeine have been shown to readily traverse cell membranes (Bianchi 1962, Bellemann & Scholz 1975, Cotgreave & Caldwell 1983) and in the intact dog intracoronary infusions of a high molecular weight theophylline derivative (too large to penetrate cell membranes) were found to antagonize adenosine but to lack direct theophylline-like cardiovascular actions (Olsson et al 1976), suggesting that theophylline may have to enter cardiovascular cells to produce its effects. However, it has been shown that the positive inotropic effect of theophylline can be dissociated from its myocardial uptake (Bellemann & Scholz 1975) and that tracheal relaxatory responses can be obtained with permanently charged xanthines (Ekman E., Kjellin G. & Persson C.G.A., unpublished observations), apparently pointing to an extracellular site of action.

It may be of interest that xanthines seem to be less potent as relaxants of K^+ -depolarized (Cerrina et al 1983, Nielsen-Kudsk J.-E., Karlsson J.-A. & Persson C.G.A., unpublished observations) than of mediator-contracted guinea-pig tracheas (cf. III). The tonic component in

K⁺-depolarized smooth muscle has been suggested to depend principally on transmembraneous influx of extracellular Ca²⁺ whereas the mediator-induced contraction rather is due to the release of Ca²⁺ from intracellular stores (Farley & Miles 1978, Creese & Denborough 1981). Thus, only at large (supratherapeutic) concentrations xanthines may have actions resembling those of the Ca²⁺-antagonists, which are more effective as relaxants of K⁺-contracted than of mediator-contracted smooth muscle (Cerrina et al 1983, Duncan & Douglas 1984, Karlsson J.-A., unpublished observations). Ca²⁺-antagonists seem to have variable effects in asthma (Patel 1981, Fanta & Drazen 1983, Henderson et al 1983). It appears therefore that the bronchoconstriction in asthma is mainly by mechanisms not involving Ca²⁺-antagonist sensitive Ca²⁺ fluxes. This possibility as well as the effects of xanthines on intracellular Ca²⁺-fluxes obviously need further attention.

As discussed above, neither inhibition of PDE activity nor adenosine antagonism, nor Ca²⁺ antagonist-like effects seem to be likely mechanisms for xanthine-induced bronchodilation. Hence, the cellular and subcellular events mediating relaxant responses to xanthines remain to be established.

Neurogenic mechanisms in the regulation of airway smooth muscle tone

Cholinergic and adrenergic mechanisms

Vagal efferent nerve fibres entering the lung terminate in ganglia located within the airways (e.g. Larsell & Dow 1933). Postganglionic cholinergic nerves innervate human airways as far distal as the terminal bronchioles (Larsell

& Dow 1933, Gaylor 1934), and normally, airways tone seems to be maintained mainly by cholinergic efferent activity since atropine produces bronchodilation in both animals and man (see Nadel 1980, Gross & Skorodin 1984).

Electrical field stimulation of the isolated trachea (V-VII) confirmed the presence of constrictive cholinergic nerves in the guinea-pig airways (e.g. Coburn & Tomita 1973, Richardson & Beland 1976), and in the hilus bronchus about 40% of the magnitude of the neurogenic contraction was atropine-sensitive (VI). Exogenously added muscarinic receptor agonists (acetylcholine, carbachol) produced the largest degrees of contraction of these airway preparations (III, IV).

Cholinergic nerve stimulation and exogenously added muscarinic receptor stimulants have been shown to contract human isolated bronchi (e.g., Hawkins & Schild 1951, Persson & Ekman 1976, Richardson & Beland 1976). It was now found that also human bronchioles vividly contracted in response to carbachol (IV). Electrical stimulation of the vagus nerve produces a diffuse constriction of the bronchial tree (see Boushey et al 1980, Nadel 1980) and with the present demonstration that human small airways are sensitive to carbachol (IV), these data support the view that cholinergic mechanisms may be of importance also in small airways contractility (cf Crompton 1968, Simonson 1977).

Postganglionic sympathetic nerves entering the lung innervate the blood vessels, the sub-mucosal glands and the parasympathetic ganglia (Richardson 1979). In keeping with the suggested predominance of relaxatory β - over constrictive α -adrenoceptors in the airways (Barnes et al 1983) addition of the sympathetic neurotransmitter nor

adrenaline (von Euler 1946) has been shown to relax guinea-pig isolated tracheas and human bronchi (e.g. Zaagsma et al 1984). Also human bronchioles were found to respond with a relaxation to noradrenaline (IV).

Adrenergic nerve fibres seem to be absent in human airways (Richardson & Beland 1976, Davis et al 1982, Taylor et al 1984). Guinea-pig tracheas, on the other hand, have a sparse adrenergic innervation (O'Donnell et al 1978) and a small adrenergic component has been demonstrated in the neurogenic inhibitory response in the trachea (e.g. Coburn & Tomita 1973). However, during a maximum neurogenic inhibition the relaxation was apparently mediated by only non-cholinergic non-adrenergic nerves (V, VI). In the guinea pig bronchi, adrenergic nerves were almost absent and no inhibitory response could be induced by electrical nerve stimulation (Grundström et al 1981, VI, VIII).

The subtype of β -adrenoceptor present in the airways is obviously of great interest. Functional studies have demonstrated the guinea-pig trachea to contain both β_1 - and β_2 -adrenoceptors (O'Donnell & Wanstall 1979, Johansson & Waldeck 1981) but human airways to contain almost exclusively adrenoceptors of the β_2 -subtype (Davis et al 1980, Goldie et al 1984b). These results are in agreement with clinical data showing that β_2 - but not β_1 -adrenoceptor stimulants produce bronchodilation in asthmatics (Löfdahl & Svedmyr 1982).

The sympathetic nervous system may affect bronchial tone, for example by diffusion of noradrenaline from adjacent vascular nerve endings or by altering transmission through parasympathetic ganglia. However, it is conceivable that circulating catecholamines (predominantly adrenaline) are the major physiological stimuli to the adrenoceptors present in the airways.

Non-cholinergic non-adrenergic mechanisms in bronchoconstriction

Hypotensive and spasmogenic effects of a biological extract from horses were first described by von Euler and Gaddum (1931) who named their extract P (now referred to as substance P). Chang & Leeman (1970) completely purified SP and found it to consist of 11 amino acids. (The amino acid sequences of SP and other tachykinins studied in this investigation are shown in Table 2.) The widespread distribution of SP in the body has initiated many attempts to reveal a possible physiological role for this peptide (see Pernow 1983) and lately SP has been considered a mediator of the non-cholinergic bronchoconstriction (see Introduction).

TABLE 2

Amino acid sequences of the tachykinins and of the two substance P (SP) antagonists studied.

Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH ₂	Substance P
Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH ₂	Physalaemin
Pyr-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH ₂	Eledoisin
Arg-pro*-Lys-Pro-Gln-Gln-trp-Phe-trp-Leu-Met-NH ₂	SP antagonist
Arg-Gln-trp-Phe-trp-Leu-Met-NH ₂	SP antagonist

* small letters indicate D-amino acid

In the present study, about 60% of the neurogenic contraction of the guinea-pig hilus bronchus was found to consist of a non-cholinergic non-adrenergic component (VI). The hilus bronchus also contracted in response to the proposed neurotransmitter SP, as well as to the tachykinins physalaemin and eledoisin (VIII). Eledoisin was significantly more potent than the other tachykinins (VIII). Moreover these peptides contracted human bronchioles with a potency similar to that of the guinea-pig hilus bronchi (IV, Finney M.J.B. & Karlsson J.-A., unpublished observations).

SP-analogues with SP antagonistic properties have been reported to inhibit selected non-cholinergic non-adrenergic neurogenic effects (Holmdahl et al 1981, Leander et al 1981, Björkroth et al 1982). Apparently supporting the suggestion that SP is the non-cholinergic non-adrenergic neurotransmitter in the airways, SP antagonists were found to antagonize the atropine-resistant bronchoconstriction in the guinea-pig hilus bronchi (VI, Lundberg et al 1983c).

However, the SP antagonists seem to be unspecific in the sense that they antagonize the effects of several tachykinins (see Rosell & Folkers 1982). Similarly, the two SP antagonists used in this investigation (Table 2) were found to antagonize tachykinin-induced contractions of the guinea-pig hilus bronchi (VIII). Interestingly, the SP antagonists were more potent as inhibitors of contractions produced by eledoisin than by SP and physalaemin (VIII). The observation that, contrary to what would be expected, SP antagonists more readily inhibited the non-cholinergic non-adrenergic contraction than the contraction produced by exogenous SP (VIII), thus indicates that another tachykinin (possibly eledoisin-like, Hua et al 1984) perhaps is a more likely neurotransmitter candidate than

SP itself. This view is supported by the recent demonstrations that different tachykinin-like peptides may be present in mammalian tissue (see Harman 1984).

Little is known about the effects of local anaesthetics on neurogenic responses in the airways, although these agents are frequently employed to evaluate the role of neural mechanisms in respiratory disorders such as asthma (e.g. Griffin et al 1982, Sheppard et al 1983). It was found that lidocaine and prilocaine inhibited the main part of the non-cholinergic non-adrenergic contractile response in the guinea-pig airways before they reached concentrations that inhibited the cholinergic response (VII). In view of the fact that the SP antagonists also showed a preference for non-cholinergic effects, the possibility that they exerted unspecific neurodepressant actions was examined in the electrically stimulated frog isolated sciatic nerve. The SP antagonists, but not SP itself, suppressed transmission in this nerve preparation and were found to be more potent than lidocaine (VIII). Furthermore, these SP antagonists inhibited transmission in nerve fibres in the rat isolated sciatic nerve (Post et al 1984). Both SP antagonists and local anaesthetics were more potent as inhibitors of the non-cholinergic non-adrenergic bronchoconstriction than of the neurotransmission (VII, VIII). It is, therefore, possible that a local anaesthetic-like action of the SP antagonists contributed to the inhibition of the non-cholinergic non-adrenergic bronchoconstriction.

To date, a large number of SP antagonists have been synthesized (see Introduction). It cannot be excluded that the introduction of the hydrophobic D-amino acid tryptophan into native SP is important for local anaesthetic-like properties since this substitution is shared by the

two SP-analogues used in the present investigation. Such a substitution seems to be common to SP-analogues with SP antagonistic properties (e.g. Leander et al 1983, Regoli et al 1984). These results suggest that the data obtained in the study of neurogenic mechanisms with the presently available SP antagonists should be interpreted with caution, particularly when large concentrations have been used. Further structure-activity work is desirable to obtain SP antagonists suitable for the evaluation of the role for SP in for example the regulation of airways tone. Taken together, the present findings suggest that the non-cholinergic non-adrenergic bronchoconstrictive neurotransmitter in the airways remains to be identified.

Non-cholinergic non-adrenergic inhibition of airways tone

The neural inhibition in human airways is mediated by a non-cholinergic non-adrenergic nervous system and much work has focused on the characteristics of this nervous system and on the identity of the neurotransmitter. Based on findings with drugs blocking the uptake of adenosine, Coleman & Levy (1974) suggested that adenosine may be the mediator of the non-adrenergic relaxation. Although some results seem to disagree with this view of a role for adenosine (Irvin et al 1982, Ito & Takeda 1982, Cameron et al 1983), the possible involvement of a purine derivative is still under consideration (Souhrada et al 1980, Satchell 1982). Another candidate for the non-adrenergic neurotransmitter is VIP, which has been shown to be present in nervous structures within the airways (Uddman et al 1978). Working with the guinea-pig trachea, Matsuzaki et al (1980) found that a VIP-like substance was released during inhibitory nerve stimulation and that both the release and the relaxatory response were abolished by

pretreatment with tetrodotoxin. Moreover, incubation of tracheas with antisera to VIP attenuated the neurogenic inhibition. Matsuzaki et al (1980) proposed VIP to be the non-adrenergic inhibitory neurotransmitter in the guinea-pig trachea.

In the present investigation exogenous VIP, but not adenosine, produced relaxations resembling those of the neurogenic inhibition in the guinea-pig trachea (V). Moreover, in the presence of a maximally relaxant concentration of either VIP, adenosine, ATP or adenine, the non-cholinergic non-adrenergic inhibition remained intact (V). The neural inhibition was also unaffected by concentrations of theophylline and indomethacin that antagonized relaxant effects of adenosine and ATP, respectively (V). These results suggest that neither VIP nor the purine derivatives are involved in non-adrenergic inhibition and would diminish the therapeutic interest in these compounds.

It is unlikely that adenosine is the non-adrenergic inhibitory neurotransmitter in man since inhalation of adenosine did not affect airflow in healthy subjects and produced bronchoconstriction in asthmatics (Cushley et al 1983). VIP has also been tried as an anti-asthmatic agent. Inhaled VIP was, however, found to be almost without effect on airways tone in man (a nebulized solution was inhaled; Bundgaard et al 1983, Barnes et al 1984) and on human bronchi in vitro (Davis et al 1982). Intravenously administered VIP caused an improvement in lung function (Morice et al 1983), which has been considered to be due to the release of catecholamines (Altieri & Diamond 1984, Barnes et al 1984, Morice et al 1984). If it really is an indirect effect, VIP, or a VIP-like peptide, would be of little therapeutic benefit as a drug in asthma.

SUMMARY AND CONCLUSIONS

Xanthines and β -adrenoceptor stimulants relaxed guinea-pig isolated tracheas with a spontaneous tone or contracted by possible asthma mediators. With an increase in the degree of contraction, theophylline caused a larger relaxation than isoprenaline and the potency ratio between these two drugs was reduced. Thus, xanthines were found to be more effective than β -adrenoceptor stimulants as relaxants of highly contracted airway smooth muscle.

Relaxant effects of enprofylline and isoprenaline were found to be unchanged in tracheas obtained from rats fed food-pellets containing large concentrations of enprofylline for 2 weeks or 6 months. Apparently, xanthines have a negligible ability to induce tolerance to themselves and to other relaxants in airway smooth muscle.

The potency of enprofylline was the same in tracheas from old and young rats, whereas that of isoprenaline was reduced in tracheas from old rats. These data might suggest that xanthines do not possess age-dependent relaxant actions in airway smooth muscle.

A human isolated small airways preparation was developed and found to respond to asthma mediators and bronchodilator drugs with rapid changes in tone. Xanthines consistently and a β_2 -adrenoceptor agonist, although less frequently, relaxed precontracted human bronchioles at concentrations considered to be clinically relevant.

A structure-activity study of xanthine derivatives in the guinea-pig trachea confirmed that both the 3- and the 1-position in the xanthine molecule are important for relaxant potency and that a large alkyl group in the

3-position increased potency considerably. Methyl substitution in the 9-position of the xanthine molecule completely abolished the relaxatory effects. By making small chemical changes in the xanthine molecule, derivatives which differ markedly in potency and pharmacodynamic profile (see below) can be produced.

In the guinea-pig trachea, no interaction could be found between the relaxant effects of theophylline and isoprenaline. In conjunction with the noticed larger efficacy of xanthines than of β -adrenoceptor stimulants in highly contracted airway preparations (see above), these data seem to agree with the view that inhibition of PDE activity is not important for xanthine-induced relaxation of airway smooth muscle. Structure-activity data indicated that the 1-position in xanthine is essential for antagonistic properties at adenosine-receptors. Together with the observation that adenosine is at best a very weak constrictor of airway smooth muscle, it is suggested that the relaxant response of xanthines is unrelated to adenosine antagonism. In spite of different cellular mechanisms involved, theophylline, enprofylline and terbutaline were shown to exert a functional antagonism to the airway constrictive effects of a large variety of possible asthma mediators. These types of bronchodilators may thus be expected to be clinically more effective than agents that specifically antagonize only one mediator.

Isolated airway preparations from guinea-pig and man contracted in response to possible asthma mediators of both neural and non-neural origin. The prostaglandines D_2 and $F_{2\alpha}$ and the leukotrienes C_4 and D_4 contracted both guinea-pig and human airways. Contractions were also produced by the tachykinins substance P, physalaemin and eledoisin, which had the same potencies in guinea-pig

bronchi and human bronchioles. Carbachol and histamine invariably produced the largest degrees of contraction. Noradrenaline consistently induced relaxant responses, indicating a role for the cholinergic, but not the sympathetic neurotransmitter, in bronchoconstriction.

Electrical nerve stimulation of the guinea-pig isolated trachea with a spontaneous tone produced a cholinergic contraction immediately followed by a pronounced relaxation, almost completely non-adrenergic in nature. In the guinea-pig hilus bronchi, neurogenic responses consisted of a contraction that was cholinergically mediated to about 40%. No inhibitory neural response was found in the hilus bronchi.

In guinea-pig tracheas with a spontaneous tone or contracted by carbachol, exogenous VIP, but not adenosine, produced a relaxatory response that mimicked the non-adrenergic inhibition. In the presence of maximally relaxant concentrations of VIP, adenosine, ATP or adenine the non-adrenergic inhibition remained intact. Neither theophylline (an adenosine receptor antagonist) nor indomethacin (an inhibitor of the relaxant effect of ATP) affected the neurogenic inhibition. It may be concluded that neither a purine derivative nor VIP is mediating the non-adrenergic inhibitory response.

The non-cholinergic neurogenic constriction in the guinea-pig isolated hilus bronchi could be inhibited by SP-analogues with SP-antagonistic properties, apparently indicating that SP may be the neurotransmitter. However, the SP-analogues were found to antagonize contractions produced by other tachykinins (eledoisin, physalaemin) more readily than those by SP and, in addition, to possess potent local anaesthetic-like properties in a frog iso-

lated nerve preparation. These data indicate that the presently available SP-antagonists may be of limited usefulness in studies of physiological roles for SP. Inferentially, the non-cholinergic bronchoconstrictive neurotransmitter remains to be identified.

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INFLUENCE OF TRACHEAL CONTRACTION ON RELAXANT EFFECTS *in vitro* OF THEOPHYLLINE AND ISOPRENALINE

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- 1 Relaxation by (-)-isoprenaline (Iso) and theophylline (Theo) was measured in guinea-pig isolated trachea, in the presence or absence of carbachol.
- 2 With basal tone or with carbachol at a concentration of 5.4×10^{-7} M, causing 70% maximal contraction, Iso and Theo relaxed the trachea to the same extent.
- 3 With carbachol concentrations of 5.4×10^{-6} M and 5.4×10^{-5} M (96% and 100% maximal contractions) Iso caused no more than 63% and 34%, respectively, of the maximum relaxation to Theo.
- 4 When calculated at 25% of the maximum Theo relaxation, the Iso/Theo potency ratio was gradually reduced from 14,160 when evaluated at basal tone to 1,560 at the highest carbachol concentration.
- 5 In combination, at their maximally effective concentrations, Theo and Iso produced no larger a relaxation than did Theo alone.
- 6 At the two highest concentrations of carbachol, concentration-response curves to Theo were virtually superimposable whether determined in the absence or the presence of Iso at its maximally effective concentration.
- 7 It is concluded that Theo causes a greater relaxation of highly contracted tracheal muscle than Iso.

Introduction

It is generally held that relaxation of bronchial smooth muscle is the most important anti-asthmatic effect of β -adrenoceptor stimulants as well as of methylxanthines (Kahn, 1907; Trendelenburg, 1912; Paterson, Woolcock & Shenfield, 1979). Theoretically, both types of drugs may act by increasing the intracellular level of cyclic adenosine 3',5'-monophosphate (cyclic AMP), through stimulation of adenylate cyclase and inhibition of phosphodiesterase, respectively. However, alternative mechanisms must be considered in particular with theophylline (Kolbeck, Speir, Carrier & Bransome, 1979; Bergstrand, 1980).

One way of examining differences between methylxanthines and β -receptor agonists is to study relaxant properties in isolated bronchi contracted to a varying extent by mediators of asthma. It is well established that the potency and degree of relaxation induced by β -adrenoceptor agonists are gradually reduced in the presence of increasing concentrations of a muscarinic agent such as carbachol (van den Brink, 1973; Buckner & Saini, 1975; O'Donnell & Wanstall, 1977). However, it is still debatable as to what extent the effects of xanthines may depend on the tonus of the airway smooth muscle (cf. Jones, Hamilton & Lefcoe, 1974; Olsson & Persson, 1976).

In the present study the relaxant effect of theophylline (Theo) was examined and compared with isoprenaline (Iso) in guinea-pig isolated tracheal preparations. The effects have been evaluated on basal tone as well as in the presence of different carbachol concentrations. In addition, some aspects of the combined drug actions have been studied.

These results have been presented in part at the Joint Meeting of the Scandinavian and German Pharmacological Societies (Karlsson & Persson, 1980).

Methods

Guinea-pigs of either sex weighing 200-400 g were used in the study. They were killed by a blow on the head and the trachea was dissected out. Two consecutive tracheal rings were carefully cut out and a thread tied to the cartilage on each side of the muscle. Rings were cut from different portions of the trachea and two to four preparations were obtained from each animal. After the cartilage had been cut between the threads, the open ring preparation was mounted in a jacketed, temperature controlled (37°C), 50 ml organ bath with Krebs solution of the following composition (mM): NaCl 118.0,

KCl 4.6, CaCl_2 2.5, MgSO_4 1.15, NaHCO_3 24.9, KH_2PO_4 1.15, glucose 5.5, with a pH of 7.4. The Krebs solution was gassed with 95% O_2 and 5% CO_2 .

Changes in muscle tension were measured isometrically via strain-gauge transducers (Grass FT03). The preparations were adjusted to an initial tension of about 0.6 g. They were allowed to rest for 30–60 min before any drugs were added. During the resting period the muscle tonus mostly increased until a stable tension was attained which varied between 0.4 and 1.2 g. Some preparations exhibited spontaneous activity but this had always ceased before the resting period was over.

The drugs were added by injection to the organ bath in a cumulative geometric progression of concentrations, until no further effect was obtained. On a total of 72 preparations, 142 concentration-response (C/R) evaluations were made. As a rule, each specific drug evaluation was done on two different rings from the same animal yielding a mean result which was used for statistical calculations. Each C/R line that is illustrated is calculated from results obtained in six to nine animals. Mean values $\pm$ s.e. mean were calculated for each concentration of the relaxant drugs. Regression analysis was made of the linear portion of the C/R lines. Differences were examined by Students' *t* test for unpaired observations.

Substances used were: carbamylcholine chloride (Ph. Nord.), (–)-isoprenaline hydrochloride (Sigma), theophylline, anhydrous (Knoll). Stock solutions were made up daily, theophylline with an additional equivalent of 0.5 M NaOH and (–)-isoprenaline with 0.1 mg/ml ascorbic acid.

Results

Contraction by carbachol

Cumulative C/R curves for carbachol were evaluated on 6 preparations (Figure 1). The calculated pD_2 value was 6.70 ± 0.11 , well in accordance with values from other types of guinea-pig tracheal preparations (Buckner & Saini, 1975; Spilker & Minatoya, 1975; Hooker, Calkins & Fleisch, 1977). Three different concentrations of carbachol, 5.4×10^{-7} M, 5.4×10^{-6} M and 5.4×10^{-5} M, were selected, corresponding to 70%, 96% and 100% of the maximum tension produced by carbachol.

Relaxation by theophylline (Theo)

Cumulative C/R curves for Theo are shown in Figure 2. With each increase in carbachol concentration the

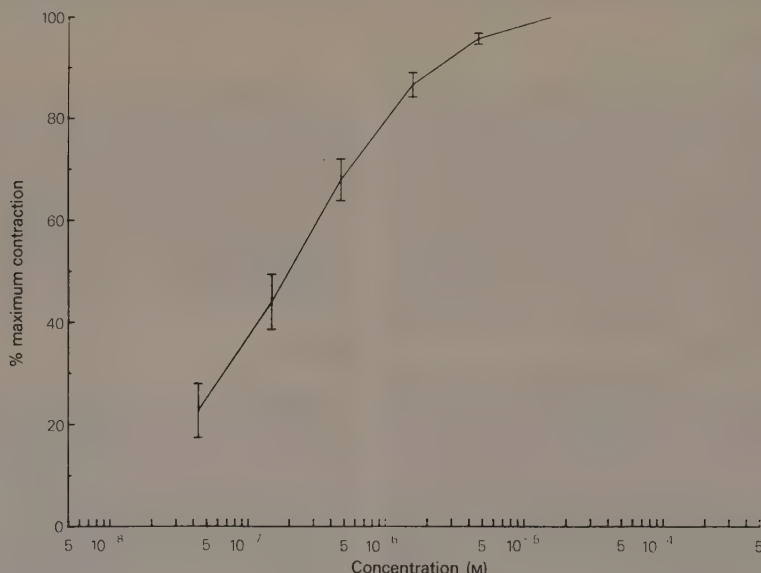


Figure 1 Cumulative concentration-response (C/R) curves for carbachol-induced contraction of guinea-pig isolated trachea. On the ordinate scale, contraction is expressed as a percentage of its own maximum. Mean results of 6 experiments are shown; vertical bars indicate s.e. mean.

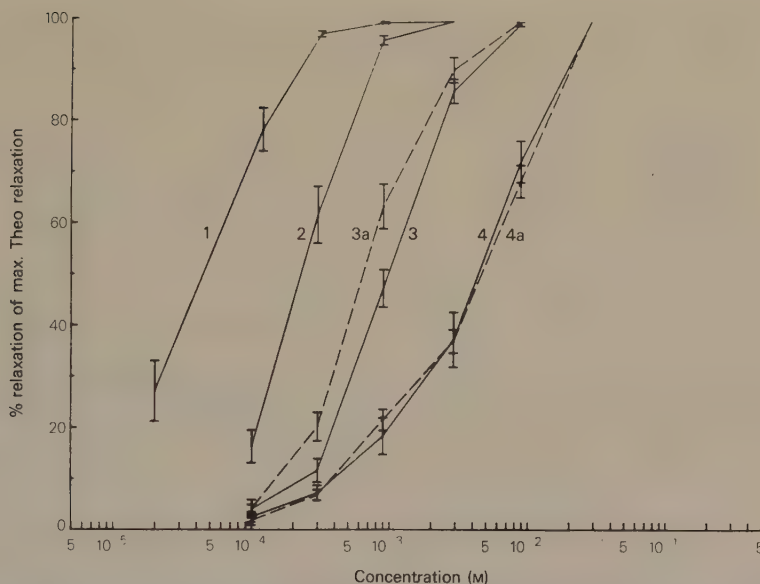


Figure 2 The complete lines illustrate cumulative concentration-response (C/R) curves for theophylline (Theo) on guinea-pig isolated tracheal muscle. The muscles were either spontaneously contracted (1) or contracted by carbachol: 5.4×10^{-7} M (2), 5.4×10^{-6} M (3) and 5.4×10^{-5} M (4). The broken C/R curves represent the effects of Theo when evaluated in the presence of a maximally effective isoprenaline (Iso) concentration. These two sets of tracheas were contracted by carbachol: 5.4×10^{-6} M (3a) and 5.4×10^{-5} M (4a). Mean results are shown; vertical bars indicate s.e. mean.

curves were shifted in parallel to the right, without any tendency of the shift to reach a maximum (Figure 2). When measured from each new level of tone, the amplitude of maximum relaxation to Theo became smaller. This change has been expressed by measuring the ratio of the size of the subsequent maximal Theo-induced relaxation to the size of the carbachol-induced contraction (see Table 1). The ratio fell from 2.0 to 1.0 as the carbachol concentration was increased from 5.4×10^{-7} M to 5.4×10^{-5} M. Addition of Iso after a maximal Theo relaxation did not produce any further relaxation at any carbachol concentration.

Relaxation by isoprenaline (Iso)

Cumulative C/R curves to Iso are shown in Figure 3. Also, in the presence of a maximally effective concentration of Iso, the C/R to Theo was evaluated. When expressing the Iso effect as a percentage of the maximum Theo relaxation (Figure 3), the curves were shifted to the right, the maximum depressed and the slopes flattened as the carbachol concentration was increased.

Iso relaxed, to the same extent as Theo, tracheal preparations with spontaneous tone and also those incubated in the low concentration of carbachol. In the presence of higher concentrations of carbachol (5.4×10^{-6} M and 5.4×10^{-5} M) Iso was less effective, its maximal effect being 63 and 34%, respectively, that of Theo. When the effect of Iso was calculated as a percentage of its own maximum, the rightward shift of the C/R lines reached a maximum at a carbachol concentration of 5.4×10^{-6} M (cf. EC_{50} values for Iso at normal pH shown in Table 3).

When measured from each new level of tone, the amplitude of maximum relaxation to Iso became smaller. The ratio, measured as for Theo described earlier, decreased from 2.0 to 0.3 as the carbachol concentration was increased from 5.4×10^{-7} M to 5.4×10^{-5} M (see Table 1).

Combined actions of theophylline and isoprenaline

In the presence of the two highest carbachol concentrations, where Theo was the more effective relaxant, the combined actions of Iso and Theo were evaluated. In preparations already relaxed by a maxi-

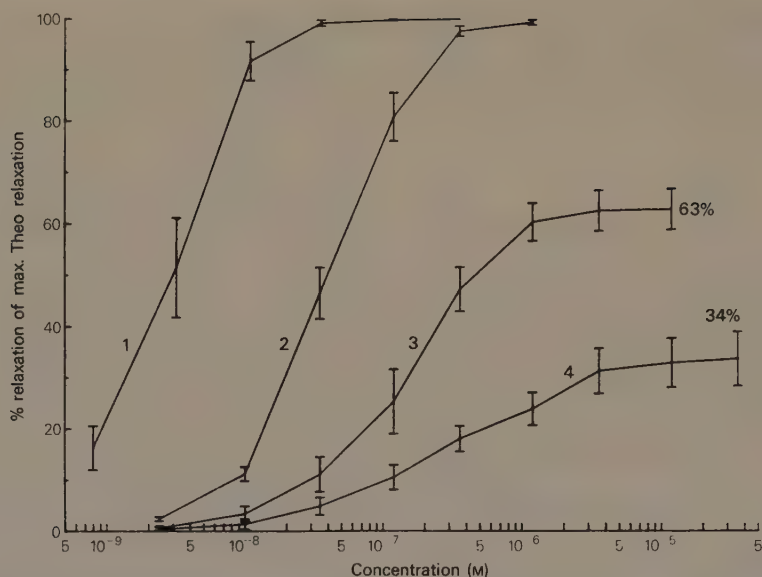


Figure 3 Cumulative concentration-response (C/R) curves for isoprenaline (Iso)-induced relaxation of guinea-pig isolated trachea. On the ordinate scale, relaxation is expressed as a percentage of the maximum theophylline effect. The effect of Iso was evaluated on either spontaneously contracted trachea (1) or on trachea contracted by different concentrations of carbachol: 5.4×10^{-7} M (2), 5.4×10^{-6} M (3) and 5.4×10^{-5} M (4). Mean results are shown; vertical bars indicate s.e.mean.

mally effective concentration of Iso, further relaxation occurred with cumulatively added Theo and the curves were almost superimposable on those caused by Theo alone (Figure 2). In the presence of the medium carbachol concentration, the C/R line for the additional relaxation by Theo was shifted leftwards 1.4 times compared to that obtained with Theo alone (confidence limits 1.08–1.7, $P < 0.01$). No shift was found with the highest carbachol concentration. The maximum relaxation with Theo and Iso combined was slightly larger than that produced by

Theo alone at the medium carbachol concentration (Table 1), whereas at the highest carbachol concentration no difference was seen.

Potency ratios between isoprenaline and theophylline

The potency ratios between Iso and Theo in different contractile states were calculated, based on concentrations of each drug producing 25% of the maximum Theo-relaxation. These ratios thus defined potency differences between Iso and Theo on the linear por-

Table 1 The ratio of the size of the maximum relaxation (g) produced by isoprenaline (Iso), theophylline (Theo) or their combination with respect to the size of the carbachol-induced contraction (g)

Carbachol (M)	Iso	n	Theo	n	Iso + Theo	n
5.4×10^{-7}	$2.0 \pm 0.2^*$	6	2.0 ± 0.4	6		
5.4×10^{-6}	1.0 ± 0.1	8	1.4 ± 0.2	8	1.6 ± 0.2	8
5.4×10^{-5}	0.3 ± 0.1	6	1.0 ± 0.1	6	1.0 ± 0	5

Mean results $\pm$ s.e.mean are given.

*A value of 1 equals complete inhibition of the contraction by carbachol. A value > 1 means that the trachea was relaxed below the basal tone that existed before the addition of carbachol. Conversely, a value < 1 indicates that the drug could not fully relax the tissue to the level of basal tone.

Table 2 Negative log molar EC₂₅ values for theophylline (Theo) and isoprenaline (Iso) on tracheas spontaneously contracted or contracted by different concentrations of carbachol

	Carbachol concentration (M)			
	0	5.4×10^{-7}	5.4×10^{-6}	5.4×10^{-5}
Theo EC ₂₅	4.66 ± 0.06	3.90 ± 0.03	3.34 ± 0.05	2.84 ± 0.09
n	8	7	9	8
Iso EC ₂₅	8.81 ± 0.09	7.79 ± 0.07	6.92 ± 0.18	6.04 ± 0.17
n	9	7	9	5
Potency ratio	14125	7762	3802	1585

Concentrations are expressed as $-\log M$ ($\pm$ s.e.mean). EC₂₅ values calculated from the drug concentration yielding 25% of the maximum effect produced by Theo.

tion of the C/R lines to both drugs and for each carbachol concentration, at the same degree of relaxation.

The Iso/Theo potency ratio was gradually reduced from about 15000 at basal tone to about 1500 at the highest carbachol concentration (Table 2).

Influence of pH on the relaxation

Theo had to be dissolved as the sodium-salt and the addition of high concentrations of Theo produced a rise in pH of the Krebs solution (Table 3). Alkalosis under *in vitro* and *in vivo* conditions has earlier been shown to affect only slightly the relaxant and contractile effects of drugs in bronchial smooth muscle (Stephens, Meyers & Cherniack, 1968; Baïsset, Montastruc & Tran, 1971; Duckles, Rayner & Nadel, 1974). It was still of interest to examine whether the increased pH influenced the present comparison between Iso and Theo.

Control experiments were thus performed with Iso in a Krebs solution where pH was elevated by addition of 0.5 M NaOH (Table 3).

There was no sign of breakdown of Iso during the alkalosis, i.e. no colour change occurred and normal, sustained relaxations were produced by Iso. The carbachol-induced contraction was not affected by

the alkalosis and the C/R curves for Iso were identical with those obtained under normal conditions (Table 3, cf. Figure 3). It was concluded that the pH-changes in the organ bath did not affect the present comparison between Iso and Theo.

Discussion

β -Adrenoceptor stimulants and methylxanthines are about equally widely used in the therapy of obstructive lung disease. In studies on drug actions, it seems that attention has been focused mainly on various aspects of β -receptor agonists. This is reflected in the abundance of studies dealing with relaxant characteristics of β -receptor agonists on bronchi (van den Brink, 1973; Raper & Malta, 1973; Buckner & Saini, 1975; O'Donnell & Wanstall, 1977). Some comparisons between β -stimulants and methylxanthines have previously been made in isolated airway smooth muscle preparations. These studies have shown that in tracheal preparations moderately contracted by various agents, β -agonists and Theo are about equally effective, with relative potencies that are proportional to the potencies found clinically (Dungan & Lish, 1961; Olsson & Persson, 1976). In addition, β -receptor stimulation did not produce a further relax-

Table 3 Negative log molar EC₅₀ ($\pm$ s.e.mean) values of theophylline and isoprenaline in relaxing carbachol-contracted tracheal muscle and corresponding pH values

Carbachol (M)	pH	Theophylline $-\log EC_{50}$	n	pH (a)	Isoprenaline $-\log EC_{50}$	n	pH (b)	$-\log EC_{50}$	n
0	7.5	4.30 ± 0.05	8	7.4	8.52 ± 0.09	9			
5.4×10^{-7}	7.6	3.64 ± 0.04	7	7.4	7.41 ± 0.06	7	7.9	7.49 ± 0.02	4
5.4×10^{-6}	7.6	3.02 ± 0.03	9	7.4	6.85 ± 0.13	9	8.2	6.93 ± 0.06	4
5.4×10^{-5}	7.8	2.42 ± 0.08	8	7.4	6.65 ± 0.22	6	9.0	6.25 ± 0.55	2

Theo pH values were measured with the corresponding concentration of Theo in Krebs solution. With Iso, EC₅₀ values were determined (a) in normal Krebs solution at pH 7.4 and (b) in Krebs solution with sufficient NaOH added to adjust pH as shown.

ant effect when added to human isolated large and small bronchi that had been relaxed by maximally effective Theo concentrations (Persson, 1980). Other workers comparing β -agonists and Theo in guinea-pig tracheas have suggested that carbachol may affect relative potency but not the degree of relaxation (Jones *et al.*, 1974). This is at variance with findings on bovine trachea where Theo fully (100%) and Iso partially (approx. 75%) relaxed the tension induced by carbachol, 4×10^{-7} M (Andersson, Kövesi & Ericsson, 1978). Working with cat isolated trachea, Mitchell & Denborough (1979) found that a high concentration of Theo (10 mM) caused a flattening of the dose-response curve to acetylcholine while a 100,000 times lower concentration of Iso ($0.1 \mu\text{M}$) only displaced the curve to the right. Thus in earlier literature there was some information suggesting that in contracted tracheas the mechanical response to β -agonists might differ from that to Theo.

Because of its use in the earlier studies with β -agonists (Buckner & Saini, 1975) it was natural to select carbachol as the contractile agent in the present study. This drug would also show the contraction characteristics of acetylcholine which may be the key mediator among all the possible non-allergic and allergic asthma mediators. The major importance of acetylcholine in asthma is indicated by observations that atropine often is clinically effective although it antagonizes this mediator only (cf. Crompton, 1968; Simonsson, 1977). The use of tracheal smooth muscle from guinea-pig was justified by findings that it may react similarly to more peripheral airways as well as to human isolated bronchi (Persson & Ekman, 1976).

At basal tone and in the presence of the low carbachol concentration, Iso and Theo were found to relax the tracheas to the same extent, Iso being about 10,000 times more potent than Theo. This is what can be expected in moderately contracted preparations (Dungan & Lish, 1961; Olsson & Persson, 1976). However, by increasing the concentration of carbachol above 5.4×10^{-7} M, the degree of relaxation by Iso, relative to that caused by Theo, was gradually reduced. At the highest carbachol concentration (5.4×10^{-5} M) Iso produced one third of the maximal relaxant response to Theo. Additionally, when

evaluated at spontaneous tone and at the progressively increasing carbachol concentrations, potency ratios between the two relaxants were gradually reduced (from about 15,000 to 1,500).

Among the β -receptor agonists, Iso is the most effective relaxant of highly contracted tracheal preparations (Raper & Malta, 1973), and a possible clinical importance of differences in effectiveness among β -agonists has been suggested (O'Donnell & Wanstall, 1978). The present findings related to Iso and carbachol are in agreement with earlier observations (Buckner & Saini, 1975). The relaxant properties of Theo were also affected by carbachol but, as discussed above, less so than Iso. The results thus showed Theo to be a more efficient relaxant of highly contracted tracheal muscle than Iso. The importance of this difference is difficult to assess. It is for instance not known if the results can be confirmed *in vivo*. Furthermore, relaxation of bronchial smooth muscle may be just one of many pulmonary effects that can be produced by β -agonists and methylxanthines. Comparisons of effectiveness of Theo and β -receptor agonists should also be of interest concerning e.g. effects on mediator-induced oedema (Persson, Ekman & Erjefält, 1979) and anaphylactic reactions in the lung (Andersson, 1980).

It seems likely that Iso may initiate relaxation of tracheal smooth muscle by stimulating adenylate cyclase and thus raising the level of cyclic AMP (Andersson, 1972; Andersson *et al.*, 1978). Theo, being a phosphodiesterase inhibitor, might also act by increasing the intracellular level of cyclic AMP but the importance of this mechanism has been seriously questioned. For instance, Kolbeck and co-workers (1979) working with guinea-pig and canine tracheal tissues showed that theophylline-induced relaxant effects were not associated with changes in the intracellular level of cyclic AMP. Our finding, that the sensitivity of the tracheal muscle to the action of Theo was unaffected whether in the presence or absence of Iso, a drug that effectively stimulates adenylate cyclase, seems to agree with the view that mechanisms of action other than phosphodiesterase inhibition are involved in the relaxant effects of Theo.

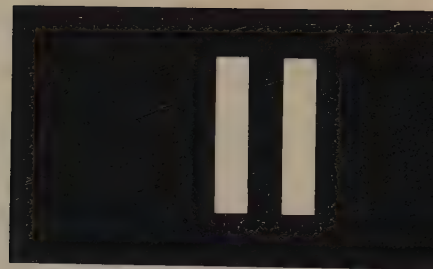
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Effects on tracheal smooth muscle of adenosine and methylxanthines, and their interaction

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The relaxant effects of xanthine and 15 different methylxanthines were studied in a guinea-pig isolated tracheal ring preparation. Substitution in the 3- and 1-positions of the xanthine molecule were found to be of greatest importance for improving tracheal relaxant potency. The unsubstituted xanthine and all 9-methylated xanthines were weakly active. Adenosine occasionally contracted (slightly) the tracheae but a concentration-dependent relaxation was always recorded. Endogenous adenosine did not seem to contribute to the tracheal tone. Xanthines methylated in the 1-position consistently antagonized the relaxant effect of adenosine and produced graded rightward shifts of the concentration-response line to adenosine (competitive antagonism). Xanthines with an unsubstituted 1-position or with comethylation in the 9-position were generally without antagonism. The results support our view that bronchodilating xanthine derivatives lacking universal adenosine antagonism can be produced.

Little is known about the biological role of adenosine in the lung. It seems that opposite actions can be produced both on smooth muscle tension and on release of histamine from various cells (Fredholm et al 1979; Marquardt et al 1978; Marone et al 1979). Theophylline at low and therapeutic concentrations antagonizes adenosine's bronchorelaxant effects but potent bronchodilator alkylxanthines have been produced that seem to lack antagonism at important adenosine receptor sites (Persson & Kjellin 1981; Persson et al 1981; Lunell et al 1982; Persson & Erjefält 1982).

The xanthine molecule can be substituted at several positions to give derivatives that qualitatively and quantitatively differ from theophylline (Bock 1920; Armitage et al 1961; Goodsell et al 1971; Bergstrand et al 1978; Persson 1980; Persson & Kjellin 1981 and unpublished observations). To examine which positions are most important for bronchodilation and antagonism of adenosine-induced relaxation, respectively, xanthine and a variety of methylxanthines have been evaluated in a tracheal preparation. Some results have been preliminarily communicated (Karlsson & Persson 1981b; Persson et al 1982).

MATERIALS AND METHODS

Guinea-pigs of either sex, 200-400 g, were killed by a blow on the head and the trachea rapidly dissected. Open tracheal rings were prepared (Karlsson & Persson 1981a) and suspended under a resting

tension of 0.6 g in 50 ml organ baths with heated (37 °C) Krebs solution (mm: NaCl 118.0, KCl 4.6, CaCl₂ 2.5, MgSO₄ 1.15, NaHCO₃ 24.9, KH₂PO₄ 1.15, glucose 5.5, pH = 7.4) and gassed with 5% CO₂ in oxygen. The preparations were left to equilibrate for about 1 h during which a stable spontaneous tension between 0.6 and 2.0 g was attained.

Isometric tension changes were measured with Grass strain-gauge transducers (FT03) and recorded on a Grass polygraph model 5. The drugs were added cumulatively by injection to the organ baths until no further effect was obtained.

Relaxant dose-response (D/R) curves were evaluated to xanthine and the methylxanthines in tracheal preparations contracted by carbachol 5.5×10^{-7} mol litre⁻¹. Intrinsic tone preparations were used in all experiments involving studies of adenosine.

D/R-curves to adenosine were evaluated in the absence and presence of different concentrations of the xanthines. Initially, in each preparation, adenosine control-curves (in the absence of xanthines) were evaluated at least twice to obtain stable tissue sensitivity to adenosine. After this protocol adenosine-induced relaxations were reproducible since EC₅₀ values from two to four D/R-curves to adenosine, obtained in each preparation during the day, generally deviated less than 10% from the mean value in that preparation.

To study adenosine-antagonism, different concentrations of the xanthines were added to the bath

* Correspondence.

before D/R-curves to adenosine were evaluated. The adenosine EC50 value obtained after pretreatment for 20 min did not differ from that obtained after 5-min contact so the xanthines were added 5 to 10 min before adenosine.

Dipyridamole at 2 $\mu\text{mol litre}^{-1}$, when added 15 min before adenosine, produced an approximately 50-fold leftward shift of the adenosine-curve. This concentration which did not affect baseline tension or D/R-curves to the xanthines, was always present when adenosine-effects were evaluated.

Mean $\pm$ s.e.m. values were calculated for each concentration of the drugs. EC50 values (the concentrations of drug producing 50% of its own maximal response under the chosen experimental condition) are mean $\pm$ s.e.m. results from each individual D/R curve. Adenosine EC50 values in the presence of xanthines were compared with mean adenosine control values from the same preparation, only. Thus, adenosine-antagonistic potency-values of each compound are the mean of concentration ratios from individual preparations. Statistical differences, based on EC50 values, were calculated by use of Student's *t*-test for unpaired observations.

Adenosine, adenosine-5'-triphosphate, carbachol, 1,3,7-trimethylxanthine (caffeine) and dipyridamole were dissolved in 0.9% NaCl (saline). Other compounds were dissolved in 1 equiv. of 0.5 mol litre⁻¹ NaOH and then diluted in saline. Drugs used were: adenosine (Sigma), adenosine-5'-triphosphate (ATP, Sigma), adenosine deaminase, EC 3.5.4.4 Type III, (Sigma), carbachol chloride (Ph. Nord.), dipyridamole (Persantin, Boehringer, Ingelheim), theophylline, anhydrous (Knoll), xanthine (Merck), 3,7-dimethylxanthine (theobromine, Ph.Nord.), 1,3,7-trimethyl-xanthine (caffeine, Ph.Nord), 1-methylxanthine, 7-methylxanthine, 8-methylxanthine, 9-methylxanthine, 1,7-dimethylxanthine, 1,9-dimethylxanthine, 3,9-dimethylxanthine and 1,3,9-trimethylxanthine (Fluka), 3-methylxanthine, 3,8-dimethylxanthine and 1,3,8-trimethylxanthine were formed by the classical Traube route (Traube 1900). 1,8-dimethylxanthine was prepared according to Cook et al (1949). Tetrodotoxin (Sankyo).

RESULTS

Tracheal relaxation by xanthine and various methylxanthines

Open guinea-pig tracheal rings, spontaneously contracted (0.6 to 2.0 g) or contracted by $5.5 \cdot 10^{-7}$ mol litre⁻¹ carbachol (corresponding to 70% of a maximum carbachol-contraction, Karlsson & Persson

1981a) were relaxed in a concentration-related way by the methylxanthines.

When repeatedly exposed to high concentrations of methylxanthines, the rings gradually lost their ability to regain a spontaneous tone after rinsing. Therefore, the statistical evaluation of relative relaxant potencies was made with carbachol-contracted rings. The rank order and degree of relaxation among the methylxanthines in a preliminary study were the same, whether evaluated at basal tone or at the chosen carbachol concentration.

Mean EC50 values for tracheal relaxation by the methylxanthines are given in Table 1. However, xanthine and compounds methylated in the 9-position produced maximum relaxations (in concentrations close to saturation) less than 50% of a maximum theophylline relaxation.

Table 1. Tracheal relaxation and adenosine-antagonism by methylxanthines. EC50-values were obtained in carbachol-contracted ($5.5 \cdot 10^{-7}$ mol litre⁻¹) tracheal rings. pA₂ values and slopes are derived from Schild plots for antagonism of adenosine-induced relaxation of intrinsic tone preparations.

Position(s) of methyl group(s)	EC50 $\pm$ s.e.m. mmol litre ⁻¹	pA ₂	Slope	95% confid. limits
-Xanthine	**	NA		
1-	1.81 $\pm$ 0.07*	4.40	1.09	0.81-1.37
3-	1.24 $\pm$ 0.11	NA		
7-	2.12 $\pm$ 0.25	NA		
8-	3.41 $\pm$ 0.21	NA		
9-	**	NA		
1,3-Theophylline	0.32 $\pm$ 0.01	4.92	1.18	0.77-1.60
1.7	0.90 $\pm$ 0.19	4.63	1.26	0.77-1.81
1.8	0.84 $\pm$ 0.02	4.95	1.01	0.51-1.51
1.9	**	NA		
3,7-Theobromine	0.83 $\pm$ 0.08	NA		
3.8-	0.60 $\pm$ 0.05	NA		
3.9-	**	NA		
1,3,7-Caffeine	0.43 $\pm$ 0.04	4.42	0.84	0.35-1.34
1,3.8-	0.30 $\pm$ 0.03	4.95	0.88	0.48-1.28
1,3.9-	**	NA		

* Each value is the mean of 4 preparations.

** Less than 50% of a maximum theophylline induced relaxation was produced.

NA - No antagonism, see text.

Among monosubstituted xanthines, methylsubstitution in the 3-position gave more active compounds than substitution in 1-, 7- or 8-position. 9-Methylxanthine was almost inactive. The disubstituted methylxanthines exhibited the following rank order of potency: 1,3>3,8>3,7>1,8>1,7. The 1,9 and 3,9-dimethylxanthine were almost inactive. The potencies among trisubstituted compounds ranked: 1,3,8>1,3,7; 1,3,9 being almost inactive (Table 1).

Substitution in the 8-position in di- and trisubstituted xanthines produced, in contrast to monosubstituted xanthines, more active compounds than substitution in the 7-position.

Consistent with this, 1,3,8-trimethylxanthine was the most active compound, closely followed by 1,3-dimethylxanthine. Mean EC₅₀ values for theophylline in intrinsic tone ($60 \pm 11 \mu\text{mol litre}^{-1}$; $n = 4$) and in carbachol-contracted ($320 \pm 10 \mu\text{mol litre}^{-1}$; $n = 4$) preparations agree with previously reported values (Karlsson & Persson 1981a).

Effects of adenosine

The first evaluation (dipyridamole absent) of adenosine occasionally yielded a slight contraction followed by a concentration-related relaxation. The contraction, when it occurred, did not show repeatability. Whether this slight contraction was affected by tetrodotoxin (TTX $3 \mu\text{mol litre}^{-1}$) was examined in four tracheae. Another four preparations were used as controls and in two an initial contraction to adenosine was recorded. Two out of the four TTX-treated tracheae also responded with an initial contraction to adenosine.

Isolated tracheal rings with spontaneous tone or contracted by carbachol were consistently relaxed by adenosine in a dose-related way when dipyridamole was present. The maximum relaxation by adenosine in intrinsic tone or in carbachol-contracted rings was 56.7 and 17.4%, respectively, of a maximum theophylline relaxation. The corresponding mean adenosine EC₅₀ values were $9.8 \pm 0.7 \mu\text{mol litre}^{-1}$ ($n = 47$) and $12.1 \pm 3.0 \mu\text{mol litre}^{-1}$ ($n = 4$). D/R curves to adenosine in intrinsic tone preparations are shown in Fig. 1 (solid lines).

To study the possibility that an endogenous tone to adenosine contributed to the contractile state of the tracheal preparation, adenosine deaminase (0.1 u ml^{-1}) was added to the spontaneously contracted preparations. No change in tone was produced. The deaminase completely abolished the relaxation to exogenously added adenosine ($100 \mu\text{mol litre}^{-1}$) but reduced only slightly the sensitivity of the preparation to the relaxant effects of ATP.

Adenosine-antagonism by xanthine derivatives

The effect of adenosine on spontaneous tone, was evaluated in the presence of different concentrations of xanthine and of methylxanthines. The drugs were used in concentrations producing up to 50% relaxation (of their own maximum) of the intrinsic tone tracheae. Additional graded relaxant responses to adenosine could always be obtained, and from results with non-blocking xanthines it was obvious that a decrease in tone (by the xanthines) itself did not influence the adenosine evaluations.

Only xanthines methylated in the 1-position consistently shifted the D/R curve to adenosine to the right and these produced concentration-related shifts, without any decrease in maximum adenosine

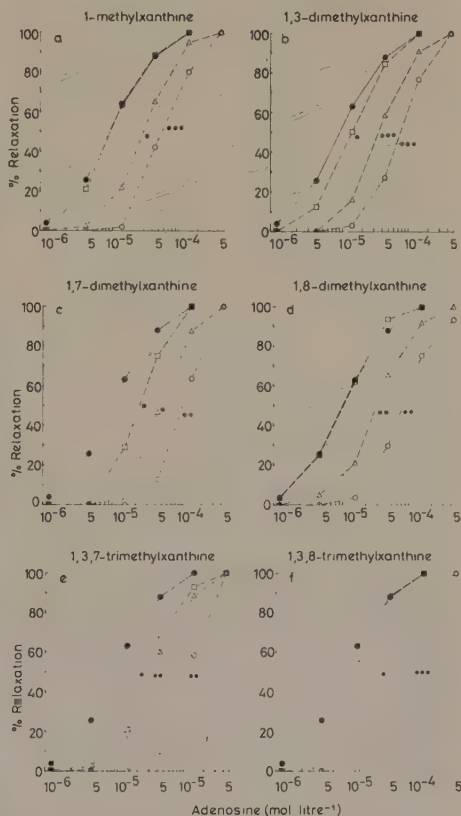


FIG. 1. Antagonism by methylxanthines of adenosine-induced relaxation of tracheal rings. Dose-response (D/R) lines to adenosine in the absence of methylxanthines shown in a-f (solid lines, ●) are mean results from 47 preparations. D/R lines to adenosine in the presence of different concentrations of methylxanthines, shown in a-f (broken line, open symbols), are mean results from 4 to 10 preparations. Adenosine effects were always studied in the presence of $2 \mu\text{mol litre}^{-1}$ dipyridamole. Statistical differences (Student's *t*-test) between mean EC₅₀ values for adenosine in the absence and presence of methylxanthine are denoted by: $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***. Doses in $\mu\text{mol litre}^{-1}$: a: 1-methylxanthine; 15 (□), 50 (Δ), 150 (○); b: 1,3-dimethylxanthine; 10 (□), 30 (Δ), 60 (○); c: 1,7-dimethylxanthine 15 (□), 50 (Δ), 150 (○); d: 1,8-dimethylxanthine, 15 (□), 50 (Δ), 150 (○); e: 1,3,7-trimethylxanthine, 30 (□), 90 (Δ), 300 (○); f: 1,3,8-trimethylxanthine, 8 (□), 23 (Δ), 69 (○).

relaxation (Fig. 1). This antagonism was not much affected by methylation in the 3-, 7- and 8-positions or combinations thereof (Table 1). However, xanthine and 9-methylated xanthines were always inactive (Fig. 2). Schild plots of the active compounds indicated that the antagonism was competitive, the slopes not differing significantly from 1 ($P > 0.05$, Table 1) (see Arunlakshana & Schild 1959). The low theobromine (3,7-dimethylxanthine) concentration ($100 \mu\text{mol litre}^{-1}$) produced a slight, but significant, increase in the adenosine EC_{50} value ($P < 0.01$), whereas the higher concentration ($150 \mu\text{mol litre}^{-1}$) was inactive in this respect. 3,8-Dimethylxanthine ($100 \mu\text{mol litre}^{-1}$) also showed a weak, but significant, ($P < 0.05$) antagonism (Fig. 2).

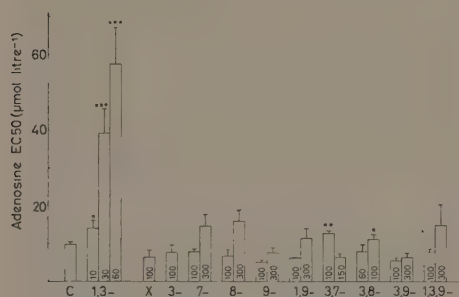


FIG. 2. Adenosine EC_{50} values in tracheal preparations in the absence or presence of 'non-blocking' xanthines. For comparison the values obtained with theophylline are included. Positions of methyl groups in the tested compounds are shown below concentration bars; xanthine is indicated by X. Concentrations of compounds ($\mu\text{mol litre}^{-1}$) are shown within bars. Adenosine control (C) EC_{50} value ($\pm$ s.e.m.) is the mean of 47 preparations. Each methylxanthine concentration was studied in 3 to 10 preparations. Adenosine EC_{50} values in the presence of the xanthines were compared statistically (Student's *t*-test) only with mean control values from the same preparations. Significant differences are denoted by $P < 0.05$, *; $P < 0.01$, **.

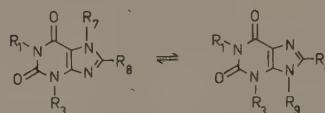
The presence of adenosine ($10 \mu\text{mol litre}^{-1}$) in the bath attenuated the theophylline-induced relaxation. The EC_{50} value of theophylline was increased about twice ($n = 4$). Addition of adenosine to markedly methylxanthine-relaxed preparations often produced a transient, slight contraction which did not impede evaluation of adenosine antagonism.

DISCUSSION

The bronchorelaxant effect of theophylline is considered to be the major therapeutic action and xanthines are effective relaxants irrespective of which mediator has contracted the bronchi (unpubli-

shed work by Karlsson, Persson and Sonmark). The degree of mediator-induced contraction will determine both the potency and efficacy of theophylline as a tracheal relaxant in vitro (Karlsson & Persson 1981a). A high sensitivity was desirable in the present study because many xanthines are poorly soluble in relation to their potency. Thus intrinsic tone tracheae or a low concentration of contractile agent were employed.

Xanthine itself and 9-methylsubstituted xanthine derivatives produced only weak relaxations. Derivatives with substitutions at other positions of xanthine were effective relaxants. In summary, the 3-position, then the 1-position, was the most important for bronchodilator potency. Excepting the monosubstituted compounds, substitution in the 8-position yielded more potent xanthines than substitution in the 7-position. In line with these observations 1,3,8-trimethylxanthine was the most potent bronchorelaxant of the methylxanthines. The activity disappears with substitution in the 9-position. With substitution in the 9-position the double bond is moved to 7-8 from the otherwise preferred location, of 8-9 (see chemical structure).



Chemical structure of the two tautomeric forms of xanthine ($R_1 = R_3 = R_7 = R_8 = R_9 = \text{H}$). The different compounds were obtained by substituting with methyl groups at the positions denoted by R.

The structure-activity findings are largely in agreement with results with xanthine derivatives substituted with groups larger than methyl (Kjellin & Persson 1980; Persson et al 1981; and unpublished observations).

In agreement with the report by Farmer & Farrar (1976), the effect of adenosine on intrinsic tone tracheae in the present study was characterized as relaxation. The preparations moderately contracted by carbachol behaved similarly. On the other hand, a slight adenosine-induced contraction has been reported to occur occasionally in tracheal preparations, although the consistent response was relaxation (Fredholm et al 1979; Persson et al 1982; Coleman 1980). In the present study, contraction was infrequent and experiments with tetrodotoxin suggested it to be a smooth muscle effect.

The degree of relaxation by adenosine was much dependent on the degree of contraction of the

muscle. In the carbachol-contracted preparations the induced relaxations reached only 20% of the maximum methylxanthine-induced effect (compared with 60% in intrinsic tone preparations).

Low and therapeutic concentrations of theophylline produce a surmountable antagonism at adenosine receptors (Rall 1980). This mechanism has been suggested to reflect its bronchodilator effect (Marquardt et al 1978; Fredholm et al 1979; Welton & Simko 1980). In guinea-pig tracheae Fredholm et al (1979) observed that adenosine produced a rightward shift of the D/R curve to theophylline. Such an interaction was shown in the present study and could be expected because in a preparation relaxed by adenosine, theophylline would produce dual opposing actions: a contraction through antagonism of adenosine and a relaxation through direct smooth muscle effects. The findings by Fredholm et al (1979), therefore, may not be interpreted as a mechanism explaining the bronchodilator effects of xanthines. Nor is it likely that adenosine contributes to the muscle tone of the isolated tracheae because addition of an adenosine catabolizing enzyme (deaminase) or blocking the cellular uptake of adenosine (dipyridamole, cf. Coleman 1976) was not associated with any change in tension. It seems that significant tracheal relaxant effects of xanthines are produced by mechanisms unrelated to an interaction with adenosine.

It can be argued that adenosine receptors mediating relaxation of bronchial smooth muscle should not be blocked, because at the concentrations reported to occur for instance in the hypoxic lung (Mentzer et al 1975), adenosine may be active as a relaxant autacoid (cf. Persson et al 1981). It is thus of both theoretical and practical interest that a potent bronchodilator xanthine (3-propylxanthine, enprofylline) has been shown to be practically devoid of antagonist activity against inhibitory effects of adenosine in smooth muscle (trachea) and nervous tissue (myenteric plexus) (Persson et al 1981, 1982). The present findings, in agreement with results obtained with enprofylline, indicate those positions of the xanthine molecule that are critical for retaining and losing, respectively, an adenosine antagonist activity in airway smooth muscle. The general finding was that the various 1-methylxanthines were potent adenosine antagonists. However, as with the bronchodilator effect, adenosine antagonist activity was lost by substitution in the 9-position. The abolition of antagonist (and relaxant) activity by alkylating the 9-position is remarkable considering that this is the position where the

purine ring is substituted in adenosine. There was no consistent result suggesting that substitution at any other position could compensate for a lack of methyl in the 1-position (cf. Fig. 2). Among the active 1-methylxanthines, there was only about a 3-fold potency difference, the most potent being 1,3,8-tri- and 1,8-dimethylxanthine and the least, 1-methylxanthine. The results suggest that in addition to methyl in the 1-position, substitutions in 3- and/or 8-positions may be favourable for adenosine antagonist potency.

The slopes of the linear Schild plot regressions for all the active antagonists were not different from 1, indicating competitive antagonism at the adenosine receptors. Despite the relatively large spread in values this result is at apparent variance with the work of Coleman (1980) who reported significant adenosine antagonism by caffeine and theophylline in guinea-pig tracheal smooth muscle but their slope values were significantly less than 1. Confirming the results by Coleman (1976) it was observed that dipyridamole $2 \mu\text{mol litre}^{-1}$ produced a 50-fold leftward shift in the dose response curve to adenosine. This high sensitivity may be a necessary prerequisite to show the antagonism by xanthines. In the absence of dipyridamole there seems to be no antagonism by theophylline and caffeine of the tracheal relaxant effects of adenosine (Jones et al 1980; unpublished work).

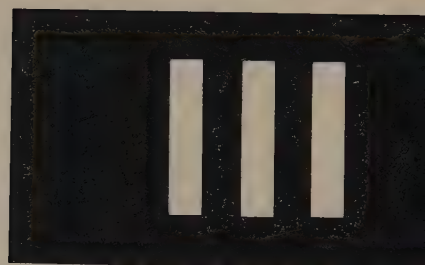
Little work has been published on structure-activity of adenosine antagonists compared with the extensive studies of adenosine receptor stimulants (cf. Baer & Drummond 1979). Scholtholt et al (1972) studied inhibitory effects of a fixed dose of different xanthine derivatives on the coronary vasodilator effect of dipyridamole. Accepting that the effect of dipyridamole reflected adenosine receptor stimulant actions, this qualitative study was the first to hint at the importance of substitution at the 1-position for adenosine blocking activity with xanthines. In apparent contrast to later work (Green & Stanberry 1977; Karlsson & Persson 1981b), Huang et al (1972) reported that 1-methylxanthine did not antagonize neuronal effects (increase in cAMP) of adenosine. When the present study was under way (cf. Karlsson & Persson 1981b) a report by Bruns (1981) appeared, showing many features of structure-activity relationships for adenosine antagonism by xanthine derivatives. Bruns, who studied adenosine antagonism by measuring the effect on fibroblast cyclic AMP concentrations, also indicated the importance of the 1-position, but among the joint 1-methylxanthines studied, the order of antagonist

potencies differed much from our findings in the trachea. We have also observed that different blocking 1-methylated xanthines are not similarly active in tracheal smooth muscle and in a myenteric plexus preparation (Karlsson & Persson 1981b, and unpublished work) which suggests that blocking xanthines may not be equally active at all adenosine receptors.

In conclusion, we have found that antagonism of the effect of adenosine on the guinea-pig trachea is unrelated to the tracheal relaxant action of xanthine derivatives. The potential value of a xanthine lacking in adenosine antagonism is illustrated by enprofylline which shares with the 1H-methylxanthines only a low ability to antagonize the inhibitory effects of adenosine in tracheal smooth muscle and enteric nervous tissue (Persson et al 1981). This 'non-blocking', 3-propylxanthine derivative is a potent bronchodilator in animals and man but seems to be devoid of theophylline-like diuretic and c.n.s.-stimulant behavioural effects (Persson & Kjellin 1981; Persson et al 1981; Persson & Erjefält 1982; Lunell et al 1982).

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ASTHMA MEDIATORS FUNCTIONALLY ANTAGONISED BY
THEOPHYLLINE, ENPROFYLLINE AND TERBUTALINE

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Abstract

Effects of possible asthma mediators have been examined in guinea-pig isolated tracheas. The mediators produced contractions with the following order of potency: $LTD_4 \geq LTC_4 > \text{carbachol} \geq \text{substance P} \geq \text{prostaglandin } F_{2\alpha} > 5\text{-hydroxytryptamine} > \text{histamine}$. The largest contractions were produced by carbachol and histamine. $PGF_{2\alpha}$ and 5-HT were the least effective mediators. Preparations treated with these mediators or with antigen were completely relaxed by a β_2 -adrenoceptor agonist (terbutaline), by an adenosine-blocking xanthine (theophylline), and by an adenosine non-blocking xanthine (enprofylline). The potency of each bronchodilator varied little in the presence of the different contractile agents. Enprofylline was consistently between 4 and 7 times more potent than theophylline, indicating that adenosine antagonism was not involved in the relaxant responses. The present data show that the two xanthines and terbutaline are effective functional antagonists of the airway constrictory action of a large variety of potential asthma mediators.

Introduction

The recent discoveries of novel types of asthma mediators have presented themselves together with the hope that the development of specific pharmacological antagonists should significantly advance the pharmacotherapy of bronchial asthma. Atropine, presumably effective by its specific antagonism of the muscarinic actions of acetylcholine, may be a prototype example of this possibility. It has not been systematically investigated whether other bronchodilator drugs owe their clinical effectiveness to selective inhibition of a particular mediator. It was thus thought of interest to compare contractions produced by several potential asthma mediators (1,2,3) and to examine the possibility that the relaxant response to xanthines, and β_2 -adrenoceptor stimulants, could depend on which type of mediator was present.

In this study effects of antigen and seven mediators including amines, a peptide, arachidonic acid products, and a muscarinic agent were examined in isolated tracheas obtained from both sensitized and normal guinea-pigs. Effects of the β_2 -adrenoceptor agonist terbutaline, the adenosine-blocking xanthine theophylline, and the adenosine non-blocking xanthine enprofylline, were evaluated in tracheas already contracted by each of the above mediators. The results have been communicated in part at a British Pharmacological Society meeting (4).

Method

Guinea-pigs (Dunkin-Hartley, body weight 180-375 g) of either sex were used. They were killed by a blow on the head and the trachea was rapidly excised and placed in cold Krebs solution. Two adjoining cartilage rings were

cut out and an open tracheal ring was prepared by cutting through the cartilage opposite to the muscle. Two to four rings were prepared from each animal. Each open ring was mounted in temperature-controlled (37°C), 10 ml organ baths containing Krebs solution (composition in mM: NaCl 118.0, KCl 4.6, CaCl_2 2.5, MgSO_4 1.15, NaHCO_3 24.9, KH_2PO_4 1.15, glucose 5.5, pH = 7.4) and gassed with 5% CO_2 in oxygen. Isometric tension changes were measured by use of strain-gauge transducers (Grass FT03) and recorded on a Grass polygraph model 5. In experiments employing leukotrienes intact tracheal rings (two cartilage rings broad) were mounted on two metal prongs in thermostated (37°C) 2.5 ml organ baths containing gassed Krebs solution (see 5). One prong was attached to a strain-gauge transducer and the other to an adjustable sled. Isometric tension changes were recorded as above.

The tension in the tracheas was initially adjusted to 0.6 g and the preparations were then allowed to stabilize for at least one hour, during which time repeated washings were performed.

Guinea-pigs were sensitized to ovalbumin (OA) by an intraperitoneal (i.p.) injection of 5 mg on day 0 and 10 mg on day 2 in 0.1 ml saline (see 6,7). Tracheas from sensitized animals were used for experiments 2 to 3 weeks later (body weight at the day of experiment was 640 to 800 g). Antigen challenge of tracheas isolated from these guinea-pigs was always examined in the presence of (pretreatment for 15-30 min) phentolamine (10^{-6}M), mepyramine (10^{-6}M) and methysergide (10^{-6}M) in order to block α -adrenoceptors, histamine H_1 -receptors and serotonin receptors, respectively. OA was evaluated only once in each preparation, either as a single concentration or in a cumulative progression of

concentrations. (The tracheas rapidly became tachyphylactic to OA).

Drugs were added by injection to the organ baths. Cumulative concentration-response (C/R) relationships were obtained by stepwise increments of the concentration in the bath until no further effect was achieved by two consecutive concentrations. Carbachol, $6 \times 10^{-8} \text{M}$, was initially added to each trachea under study in order to get a control contraction within each preparation.

Relaxant C/R relationships to theophylline, enprofylline and terbutaline were obtained in tracheal preparations which had been contracted by one of the various mediators studied. The concentration of each mediator used was that which gave an average tracheal contraction comparable to carbachol's EC50.

From C/R relationships obtained in preparations from at least 4 different guinea-pigs geometric mean $\pm$ SEM values of contractile or relaxant responses were calculated for each concentration of a drug. The mean $\pm$ SEM values are reported in the Results section. The pD_2 value is defined as the negative logarithm of the molar concentration of a drug producing 50% of its own maximum effect. For each bronchodilator, pD_2 values obtained in the presence of the different constrictors were compared using a one-way analysis of variance. If the over-all F-test was significant, pairs of pD_2 values were compared with Student's t-tests using the estimated standard deviation from the analysis of variance.

Drugs used were: atropine hydrochloride (Apoteksbolaget), carbachol chloride (Sigma), enprofylline (Draco), histamine dihydrochloride (Sigma), 5-hydroxytryptamine (5-HT,

Sigma), leukotriene C₄ (LTC₄, Merck), leukotriene D₄ (LTD₄, Merck), mepyramine maleate (May & Baker), ovalbumin grade III (OA, Sigma), phentolamine (Regitin, CIBA), prostaglandin F_{2α} (PGF_{2α} Amoglandin, Recip), substance P (SP, Sigma), terbutaline sulphate (Draco) and theophylline, anhydrous (Knoll). Theophylline and enprofylline were dissolved in 1 eqv. 0.5 M NaOH before dilution with Krebs solution. All other drugs were dissolved in and diluted with Krebs solution. Drug solutions were prepared immediately before use.

Results

Contractile effects of mediators and antigen.

The tracheal ring preparations had a marked intrinsic tone. Immediately before C/R curves to the constrictors were obtained the mean(±SEM) spontaneous tone was 0.75±0.06 g (n=31). Carbachol induced the largest degree of tracheal contraction of the substances studied. A maximum concentration ($5 \cdot 10^{-6}$ M) contracted tracheas from a spontaneous tension of 1.0±0.1 g to 1.8±0.2 g (n=4). The mean pD₂ value for carbachol was 7.14±0.03 (n=4), which is close to that earlier reported by us (8). Contractile C/R relationships were also obtained with the following substances (mean±SEM pD₂ values are shown within brackets): LTD₄ (8.60±0.25, n=5) ≥ LTC₄ (8.14±0.18, n=6) > carbachol (7.14±0.03, n=4) ≥ SP (7.11±0.11, n=4) ≥ PGF_{2α} (7.07±0.15, n=4) > 5-HT (6.64±0.20, n=4) > histamine (5.90±0.11, n=4).

In Fig 1 contractile responses to each agonist are expressed as a percentage of a maximum carbachol ($5 \cdot 10^{-6}$ M) contraction. The largest degrees of contraction were produced by carbachol and histamine whereas PGF_{2α} was the

least effective contractile agent. Apparently, the degree of spontaneous tone and the increase in contraction produced by drugs were inversely correlated; with a larger basal tension a smaller additional increase was produced. It was also observed that potency and maximum tension generated by the contractants were unrelated characteristics (Fig 1).

Tracheal rings prepared from OA-sensitized guinea-pigs developed a spontaneous tension comparable to that in non-sensitized tracheas. These preparations contracted stepwise to increasing concentrations of OA (in the presence of various antagonists, see Method). C/R relationships to OA were obtained from a mean spontaneous tension of 1.3 ± 0.2 g ($n=4$). In these tracheas, the negative logarithm of the OA concentration (g/ml) producing 50% of a maximum OA contraction was 8.61 ± 0.11 ($n=4$). A maximum contraction of 2.0 ± 0.1 g ($n=4$) was produced by $2.1 \cdot 10^{-6}$ g/ml OA. (Carbachol, $1.0 \cdot 10^{-5}$ M, added after a complete C/R evaluation to OA produced an additional contraction, Fig 1).

In order to study bronchodilators' relaxant effects the tracheas were pre-contracted with a concentration of agonist selected to produce about the same tracheal tension as $6 \cdot 10^{-8}$ M carbachol (1.4 ± 0.1 g, $n=4$; determined from the individual C/R curves). In experiments with bronchodilators the mean tensions immediately before addition of relaxatory agents were the following: carbachol ($6 \cdot 10^{-8}$ M) 1.54 ± 0.05 g ($n=14$), histamine ($1 \cdot 10^{-6}$ M) 1.24 ± 0.07 g ($n=12$), $\text{PGF}_{2\alpha}$ ($1.8 \cdot 10^{-6}$ M) 1.34 ± 0.05 g ($n=14$), 5-HT ($2 \cdot 10^{-7}$ M) 1.20 ± 0.07 g ($n=12$), SP ($3.7 \cdot 10^{-6}$ M) 1.29 ± 0.06 g ($n=12$), LTC₄ ($1 \cdot 10^{-8}$ M) 1.63 ± 0.24 g ($n=14$) and LTD₄ ($1 \cdot 10^{-8}$ M) 1.61 ± 0.20 g ($n=14$). In sensitized animals, relaxatory effects were examined in tracheas contracted by $1 \cdot 10^{-7}$ g/ml OA, producing a mean tension of 1.58 ± 0.06 g ($n=12$).

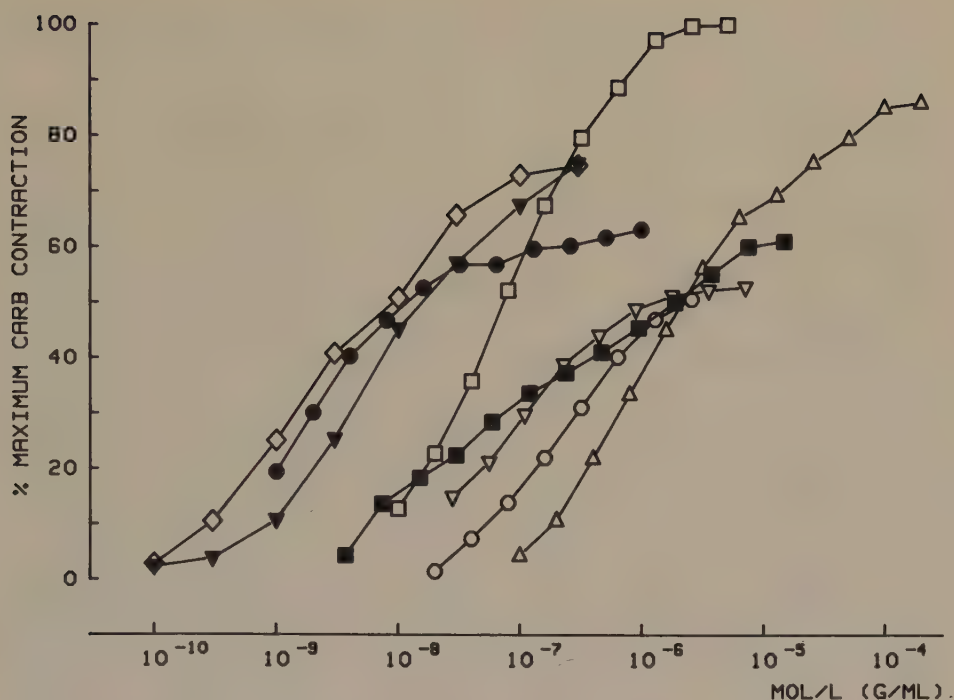


FIGURE 1

Contractile concentration-response curves for putative asthma mediators in guinea-pig isolated tracheal preparations. Tracheas were contracted by cumulative additions of carbachol (□, n=4), histamine (△, n=4), prostaglandin F_{2α} (▽, n=4), 5-hydroxytryptamine (○, n=4), substance P (■, n=4), leukotriene C₄ (▼, n=6), leukotriene D₄ (◇, n=5) or ovalbumin (●, n=4; sensitized animals). Concentrations of mediators are expressed in mol/l and of antigen in g/ml. The response to each constrictor is expressed as per cent of a maximum carbachol contraction (10⁻⁵ M). SEM ≤ 8.7% in each point and n equals number of animals studied.

Relaxation of both mediator-induced and spontaneous tone

Terbutaline (10^{-8} M to $2.6 \cdot 10^{-6}$ M), theophylline (10^{-5} M to $2.6 \cdot 10^{-3}$ M) and enprofylline (10^{-6} M to $5.1 \cdot 10^{-4}$ M) produced concentration-dependent relaxations in all contracted tracheal preparations (Fig 2). Enprofylline was consistently between 4 and 7 times more potent than theophylline. A maximum concentration of each bronchodilating drug almost completely inhibited the tracheal tone. Only 5-30% (range) of the total tone remained showing that both the mediator-induced and the spontaneous tone were inhibited. In a preparation which had received a maximum dose of one bronchodilator no further relaxant effect was produced when another bronchodilator was added. The average concentrations of terbutaline, theophylline and enprofylline needed to produce 50% inhibition of tracheas pretreated with the various constrictors differed less than 3.0, 4.2, and 6.0 times, respectively (Table 1). Analysis of variance indicated that statistically significant differences existed between mean pd_2 values for theophylline ($F=9.06$, $P<0.001$) and enprofylline ($F=12.55$, $P<0.001$) but not for terbutaline ($F=2.26$, $P>0.05$) (Table 1). By use of the Student's t-test it was found that the two xanthines inhibited the effects of 5-HT and the leukotrienes more readily the effect of carbachol (Table 1). Also with terbutaline, 5-HT-induced contractions were the most readily inhibited although this preference did not reach statistical significance.

Relaxation of only the mediator-induced tone

With the various mediators mean tracheal tension was increased between 0.2 g and 0.8 g above the spontaneously developed tone. These specific mediator- or antigen-induced contractions were inhibited by 10^{-8} M to $1.6 \cdot 10^{-7}$ M

Table 1

Relaxant PD_2 ($\pm$ SEM) values (neg. log. molar EC50 value) calculated from cumulative concentration-response curves for theophylline (THEO), enprofylline (ENPRO) and terbutaline (TERB) in guinea-pig tracheas contracted by carbachol (CARB, $6 \cdot 10^{-8}$ M), histamine (HIST, $1 \cdot 10^{-6}$ M), prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$, $1.8 \cdot 10^{-6}$ M), 5-hydroxytryptamine (5-HT, $2 \cdot 10^{-7}$ M), substance P (SP, $3 \cdot 7 \cdot 10^{-6}$ M), leukotrienes C_4 and D_4 (LTC $_4$, LTD $_4$, each $1 \cdot 10^{-8}$ M) and ovalbumin (OA, $1 \cdot 10^{-7}$ g/ml to sensitized animals).

	CARB	HIST	PGF $_{2\alpha}$	5-HT	SP	LTC $_4$	LTD $_4$	OA
THEO	4.07 $\pm$ 0.06	4.29 $\pm$ 0.11	4.08 $\pm$ 0.05	4.42 $\pm$ 0.08*	3.83 $\pm$ 0.05	4.45 $\pm$ 0.11**	4.38 $\pm$ 0.09*	3.86 $\pm$ 0.09
n	6	4	4	4	4	5	5	5
ENPRO	4.73 $\pm$ 0.09	4.90 $\pm$ 0.13	4.70 $\pm$ 0.06	5.21 $\pm$ 0.03**	4.69 $\pm$ 0.07	5.22 $\pm$ 0.08**	5.20 $\pm$ 0.09**	4.44 $\pm$ 0.05
n	4	4	4	4	4	5	5	3
TERB	7.23 $\pm$ 0.05	7.42 $\pm$ 0.08	7.17 $\pm$ 0.12	7.54 $\pm$ 0.05	7.07 $\pm$ 0.05	7.22 $\pm$ 0.14	7.35 $\pm$ 0.09	7.12 $\pm$ 0.14
n	4	4	6	4	4	4	4	4

n=number of animals studied

* denotes level of significance (compared to PD_2 value in the presence of CARB;

=P<0.05, *=P<0.01 (Student's t-test).

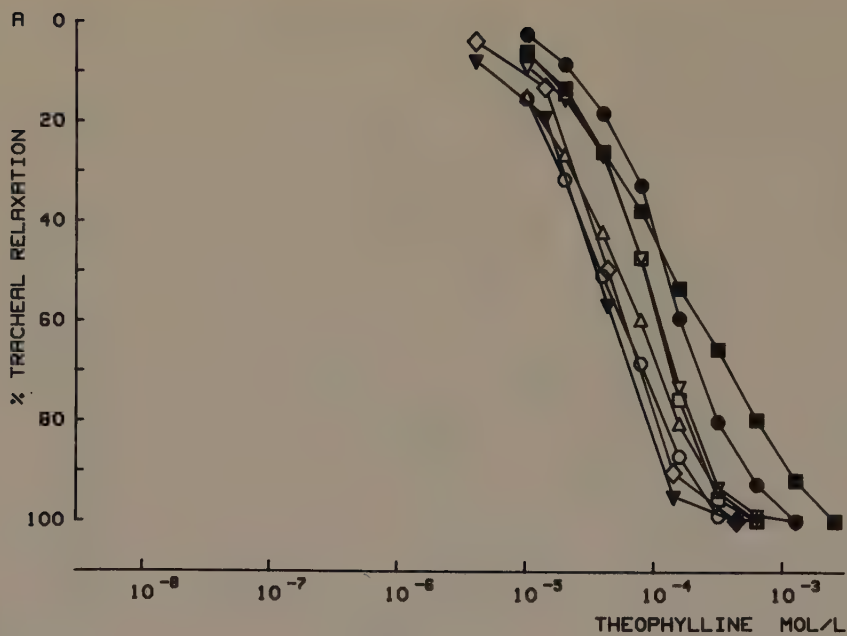


FIGURE 2 a

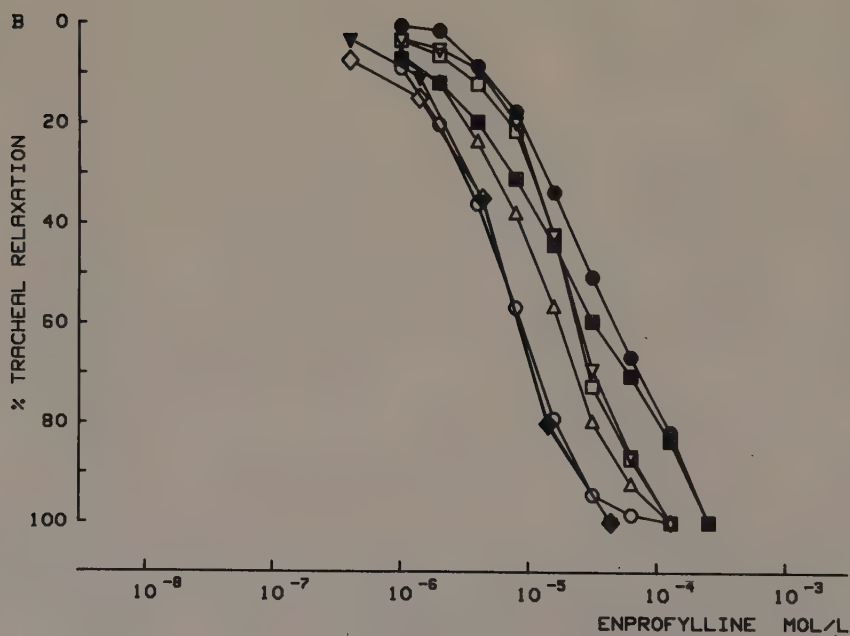


FIGURE 2 b

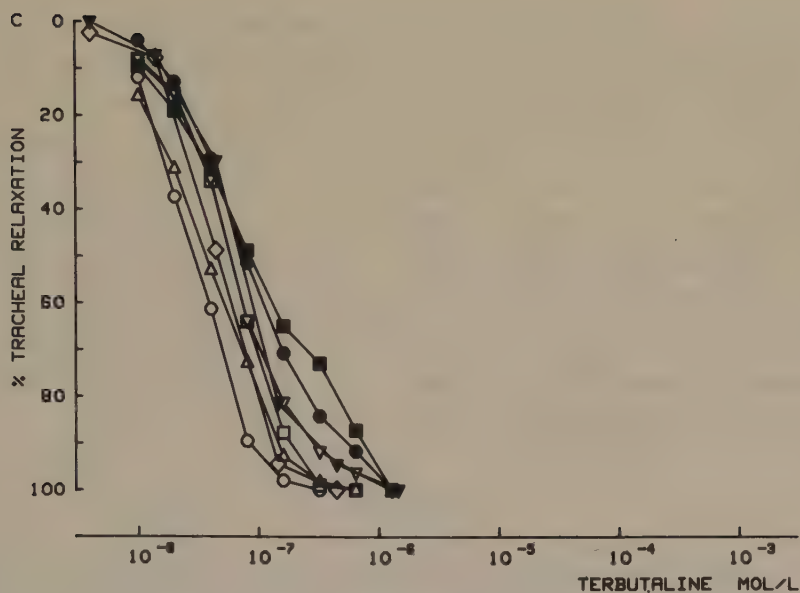


FIGURE 2 c

Legend to figures 2 a, b, c

Relaxatory concentration-response (C/R) determinations for xanthine derivatives and a β_2 -adrenoceptor agonist in guinea-pig isolated tracheas contracted by the putative asthma mediators carbachol ($\square$, $6 \cdot 10^{-8}$ M), histamine ($\triangle$, $1 \cdot 10^{-6}$ M), prostaglandin $F_{2\alpha}$ (∇ , $1.8 \cdot 10^{-6}$ M), 5-hydroxytryptamine ($\circ$, $2 \cdot 10^{-7}$ M), substance P ($\blacksquare$, $3.7 \cdot 10^{-6}$ M), leukotriene C_4 ($\blacktriangledown$, $1 \cdot 10^{-8}$ M), leukotriene D_4 ($\diamond$, $1 \cdot 10^{-8}$ M) or ovalbumin ($\bullet$, 10^{-7} g/ml; sensitized animals). Relaxant drugs were added cumulatively to pre-contracted tracheas and the effect is expressed as a percentage of its own maximum response. n (see below) equals number of animals used. a) C/R curves for theophylline, $n=4-6$; $SEM \leq 10.7\%$ in each point. b) C/R curves for enprofylline, $n=3-5$; $SEM \leq 11.7\%$ in each point. c) C/R curves for terbutaline, $n=4-6$; $SEM \leq 12.0\%$ in each point.

terbutaline, 10^{-5}M to $3.2 \cdot 10^{-4}\text{M}$ theophylline or 10^{-6}M to $3.2 \cdot 10^{-5}\text{M}$ enprofylline. Mean concentrations of the bronchodilators inhibiting the mediator-induced tone by 50% (=IC50) are shown in Table 2. The analysis of variance showed that differences existed between mean IC50 values for enprofylline ($F=5.05$, $P<0.01$) and it was found that the IC50 values obtained in the presence of leukotrienes were significantly lower than those in the presence of carbachol (Table 2). No significant differences were found between average IC50 values for theophylline ($F=2.05$, $P>0.05$) and terbutaline ($F=0.87$, $P>0.05$).

Discussion

The contractile characteristics of seven proposed asthma mediators and antigen were examined in guinea-pig tracheas with a spontaneous tone. Carbachol, mimicking effects of the parasympathetic neurotransmitter acetylcholine, produced the largest degree of contraction. The leukotrienes were the most potent constrictors (see 9,10). LTC_4 and LTD_4 were about one order of magnitude more potent than carbachol and SP, the other mediators were less potent. LTC_4 and LTD_4 are the principal constituents of slow reacting substance of anaphylaxis (SRS-A; 11,12). Release of SRS-A was most likely induced in the Schultz-Dale reaction initiated by the addition of antigen to tracheas from sensitized animals (e.g. 7,13). Although the antigen-induced contraction had characteristics similar to those of the leukotrienes, other as yet unknown mediators could well have been released and contributed to the response (14).

Non-cholinergic neurogenic contraction of airways may be mediated by SP or by a related tachykinin peptide (15,16). SP was equipotent to, but only about half as effective as,

Table 2

Negative log. molar concentrations ($\pm$ SEM) of bronchodilators inhibiting specifically a mediator-induced contraction (increase in tension above the spontaneous tone) of guinea-pig isolated tracheas. Theophylline (THEO), enprofylline (ENPRO) and terbutaline (TERB) were cumulatively added to tracheas contracted by carbachol (CARB), histamine (HIST), 5-hydroxytryptamine (5-HT), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), substance P (SP), leukotrienes C_4 and D_4 (LTC_4 , LTD_4) or ovalbumin (OA-sensitized animals) (concentrations of contractants are shown in Table 1).

	CARB	HIST	$PGF_{2\alpha}$	5-HT	SP	LTC_4	LTD_4	OA
THEO	4.61 $\pm$ 0.03	4.34 $\pm$ 0.17	4.60 $\pm$ 0.12	4.57 $\pm$ 0.04	4.22 $\pm$ 0.27	4.89 $\pm$ 0.25	5.01 $\pm$ 0.39	4.45 $\pm$ 0.10
n	6	4	4	4	4	4	3	4
ENPRO	5.26 $\pm$ 0.12	5.24 $\pm$ 0.20	4.99 $\pm$ 0.09	5.55 $\pm$ 0.11	5.44 $\pm$ 0.23	6.06 $\pm$ 0.25*	6.11 $\pm$ 0.17*	5.26 $\pm$ 0.12
n	4	4	4	4	4	5	4	3
TERB	7.61 $\pm$ 0.05	7.68 $\pm$ 0.14	7.55 $\pm$ 0.11	7.75 $\pm$ 0.10	7.43 $\pm$ 0.17	7.41 $\pm$ 0.13	7.77 $\pm$ 0.24	7.68 $\pm$ 0.19
n	4	4	4	5	4	4	4	3

n equals number of animals used

* denotes level of significance (see Table 1).

carbachol. $\text{PGF}_{2\alpha}$ and 5-HT had characteristics similar to those of SP, whereas histamine was more than one order of magnitude less potent (see 17,18,19). Thus, unknown mediators and mediators classified as amines, a peptide, arachidonic acid products, and a muscarinic agent produced significant contractions of the guinea-pig trachea. Their potencies differed as well as the magnitude of their maximum contractions. This observation would suggest that different mechanisms are involved. Apart from specific receptor activation by many of the mediators, the various biochemical pathways involved in the contractions of tracheal smooth muscle are unknown.

It is generally thought that the subcellular basis for the relaxant actions of both β -receptor agonists and xanthines involves increased cytoplasmatic concentrations of cyclic AMP, which via a number of steps leads to inhibition of the myosin-actin interaction and so contraction is prevented or reversed. Considering this mode of action it would be natural to expect these drugs to be effective against most types of contractile agents. However, the subcellular mechanisms of action of the bronchodilator drugs are still debated. It seems, in particular, that little is settled concerning the actions of xanthines (20). Furthermore, actual data are scarce on interactions between the bronchodilator drugs and the many mediators that hitherto have been put forward as potential asthma mediators. Highly contracted tracheas may not be much relaxed by β -receptor stimulants (8) and in order to compare relaxant effects the degree of contraction induced by the various mediators should not differ too much (8,21,22). Hence, in this study the relaxant characteristics of terbutaline, theophylline and enprofylline were evaluated in tracheas moderately contracted to approximately the same degree.

Spilker and Minatoya (23), working with guinea-pig trachea, reported that isoprenaline inhibited contractions by histamine more readily than those by potassium. The difference in isoprenaline potency reached almost two orders of magnitude suggesting that β -receptor agonist actions on airways may indeed depend on the type of contractile stimuli present. However, terbutaline was in the present study an effective relaxant and had an almost unchanged potency irrespective of which mediator or antigen had contracted the trachea. The tracheal tone was almost completely abolished by terbutaline indicating that also the spontaneously developed tone was inhibited. The spontaneous tone has been proposed to be due to cyclooxygenase products since indomethacin relaxes spontaneously contracted tracheas (17,19). Addition of inhibitors of the arachidonate lipoxxygenase pathway also attenuated the intrinsic tone of tracheal preparations (unpublished observations by Lundblad, Karlsson & Persson) suggesting that additional mechanisms may contribute to the basal tone. Thus, terbutaline readily relaxed tracheal tone induced by known and partly unknown (spontaneous and antigen-induced tone) mediators showing its ability to exert a functional antagonism (see 24,25). These results suggest that β -adrenoceptor mediated relaxation is little influenced by different types of constrictive agents, potentially contributing to bronchoconstriction in asthma.

Theophylline and enprofylline were also effective relaxants of the pre-contracted tracheas, inhibiting both the mediator induced and the spontaneous tone. These results tally with previous observations which have shown the xanthines to relax airway smooth muscle (see 20). Enprofylline was consistently between 4 and 7 times more potent than theophylline in agreement with the potency ratio of about 5 observed in human airways in vitro (5) and in vivo

(e.g. 26). Each of the xanthines had pD_2 values that were within a narrow range indicating that functional antagonism was exerted.

Lower concentrations of the xanthines were needed to inhibit contractions produced by 5-HT and the leukotrienes than contractions produced by carbachol. It has been shown that theophylline is a potent antagonist of effects produced by the purine nucleoside adenosine (see 20) including the adenosine-induced bronchoconstriction both in human asthmatics (27) and in the guinea-pig isolated perfused lung (28). If adenosine contributes to the actions of 5-HT and the leukotrienes the preferential inhibition of these mediators would be explained. It is, however, not likely that adenosine antagonism was expressed in the present study because adenosine itself is a relaxant rather than a constrictor of guinea-pig tracheal smooth muscle (29). Secondly, the adenosine non-blocking xanthine enprofylline, like theophylline, appeared to have a preferential action against the leukotrienes and 5-HT.

The consistent potent actions of enprofylline in this study together with clinical data (e.g. 26) suggest that functional antagonism rather than specific adenosine-receptor antagonism can explain the antiasthmatic efficacy of xanthines. The subcellular basis for functional antagonism of xanthines has not been established but remains a challenge for future research. The possibility that the xanthines selectively antagonize 5-HT and leukotriene-induced effects may also warrant further investigations.

In conclusion, three bronchodilator drugs representing β_2 -adrenoceptor agonists and different classes of xanthines all exhibited functional antagonism to the airway constrictive effects of a large variety of possible asthma

mediators and can, therefore, be expected to be effective bronchodilators irrespective of which asthma mediator has constricted the airway. In the development of novel bronchodilator drugs it may be preferable to look for compounds with functional antagonism rather than developing agents which exert specific antagonism to a single mediator.

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IV

MEDIATORS, NEUROTRANSMITTERS AND BRONCHODILATORS IN
THE REGULATION OF HUMAN SMALL AIRWAYS CONTRACTILITY

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Summary

1. Human lung bronchiolar segments (diameter 0.6-1.5 mm) were dissected and circular muscle tension recorded. The airways nature of the preparations was verified by their relaxant responses to noradrenaline (10^{-7} - 10^{-5} M) and by histology.

2. Adenosine (10^{-6} - 10^{-4} M) produced only very weak contractions whereas carbachol ($EC_{50}=4.0 \cdot 10^{-7}$ M), histamine ($EC_{50}=6.3 \cdot 10^{-7}$ M), prostaglandin D_2 ($EC_{50}=4.1 \cdot 10^{-7}$ M), substance P ($EC_{50}=4.6 \cdot 10^{-6}$ M) and ATP (10^{-6} - 10^{-4} M) produced significant contractions.

3. The contractions generally developed rapidly and were stable. The mean maximum increase in tension achieved with the most efficacious constrictor, carbachol, was 0.5 g. ATP was the least efficacious producing only about 40% of carbachol's maximum.

4. Established and putative neurotransmitters as well as mast cell derived mediators may thus contribute to rapid peripheral airways narrowing in lung disease.

5. Carbachol ($2 \cdot 10^{-6}$ M= EC_{70}) was used to produce a tension level upon which the effects of moderate concentrations of a β_2 -adrenoceptor

stimulant (terbutaline) and two xanthines (theophylline, enprofylline) were evaluated. Terbutaline ($3 \cdot 10^{-9}$ - $3 \cdot 10^{-6}$ M) relaxed 4 out of 11 bronchioles. Theophylline (10^{-5} - $4 \cdot 10^{-3}$ M) and enprofylline (10^{-6} - $4 \cdot 10^{-4}$ M) consistently relaxed the bronchiolar preparations including those exhibiting little responsiveness to the β_2 -adrenoceptor agonist.

6. Since the xanthine enprofylline, which does not block adenosine, was a relaxant five times more potent than the adenosine antagonist theophylline and since adenosine produced only weak contractions adenosine antagonism may not be important for small airways relaxation by xanthines in asthma.

7. It is suggested that the present data, which apparently differ from those obtained with lung parenchymal strips, are of relevance for human small airways reactivity.

Introduction

In chronic airway obstruction, emphysema and chronic bronchitis, the increased resistance to airflow seems to be located principally in small airways with a diameter of less than two mm. The obstruction may depend on mucus plugging, inflammatory changes including loss of tissue elastic recoil, and on bronchiolar contraction (Bignon, Khoury, Even, Andre & Brouet 1969, Bignon, Andre-Bougaran & Brouet 1970, Butler 1982, Lopez-Vidriero & Reid 1983). Also in asthma, and particularly during early pathological changes (Hogg, Macklem & Thurlbeck 1968), a considerable degree of resistance to flow is localized to small airways (Despas, Leroux & Macklem 1972, McFadden, Ingram, Haynes & Wellman 1977). McFadden & Ingram (1979) suggested

that small airways narrowing in asthma may be due to rapid and reversible changes in the contractile state of the smooth muscle. Thus, the potential importance of small airways function in lung diseases is a strong impetus for the study of small airways contractility in vitro.

Using human and animal lung specimens Persson & Ekman (1976) dissected tubal segments of small airways (about 1 mm in diameter) and showed them to be responsive to mediators and drugs with concentration-dependent tension changes (Persson & Ekman 1976, Persson 1980). Lulich, Mitchell & Sparrow (1976), on the other hand, excised strips of subpleural lung parenchyma which also were demonstrated to be responsive to pharmacological stimuli. Presumably due to the ease by which animal and human lung parenchymal strips can be prepared, this latter type of preparation has since been frequently utilized in experimental pharmacology and physiology. The results obtained have generally been interpreted as reflecting changes in peripheral airway smooth muscle tension. This is unfortunate because lung parenchymal strips contain several components with contractile properties. In addition to airway smooth muscle, vascular smooth muscle, interstitial cells (Kapanci, Assimacopoulos, Irle, Zwahlen & Gabbiani 1974) and alveolar duct smooth muscle (Miller 1921) would be able to participate in the contractile activity. Furthermore, the relative proportions of these cellular components have been shown to be highly variable in individual lung parenchymal strips (Bertram, Goldie, Papadimitriou & Paterson 1983, Finney, Berend & Black 1984).

In the present study the technique described by Persson & Ekman (1976) has been further developed and the effects of proposed asthma mediators on bronchiolar rings have been

examined. Thus, human small airways responses to histamine, prostaglandin D_2 (PGD_2), adenosine, adenosine 5'-triphosphate (ATP), substance P, noradrenaline and carbachol were evaluated. In addition, the relaxant characteristics of the β_2 -adrenoceptor stimulant terbutaline and the xanthine derivatives, theophylline and enprofylline, were examined. Part of these results have been presented to a British Pharmacological Society meeting (Finney, Karlsson & Persson 1983b).

Methods

Lung tissue resected during lobectomy or pneumonectomy, performed for localised lung lesions, was examined by a pathologist and macroscopically normal tissue was provided for use in these experiments. Within 1 hour of resection the tissue was immersed in Krebs solution (NaCl, 118.0; KCl, 4.6; $CaCl_2$, 2.5; $MgSO_4$, 1.15; $NaHCO_3$, 24.9; KH_2PO_4 , 1.15, glucose, 5.5; pH = 7.4) aerated with a mixture of 5% carbon dioxide in oxygen (carbogen) and immediately transported to the laboratory. The tissue was usually stored overnight at 4° C for use the following day.

Airways were initially identified macroscopically and a flexible plastic tube (outside diameter 0.6 mm) was gently inserted as far as possible into the airway lumen. The airway was then carefully dissected free of surrounding tissue (the plastic tube ensuring correct identification to the airway's distal end) yielding isolated bronchioles of lumen 0.6-1.5 mm. These were cut into 2 mm long tubular segments and each segment slid over two very fine wire prongs in a 2.5 ml organ bath containing Krebs solution (37° C) and gassed with carbogen. One prong was connected to a force-displacement transducer (Grass FT 03) and the other to an adjustable sled (Fig. 1). Isometric tension

was recorded on a Grass polygraph model 5 and resting tone established at 0.5 g.

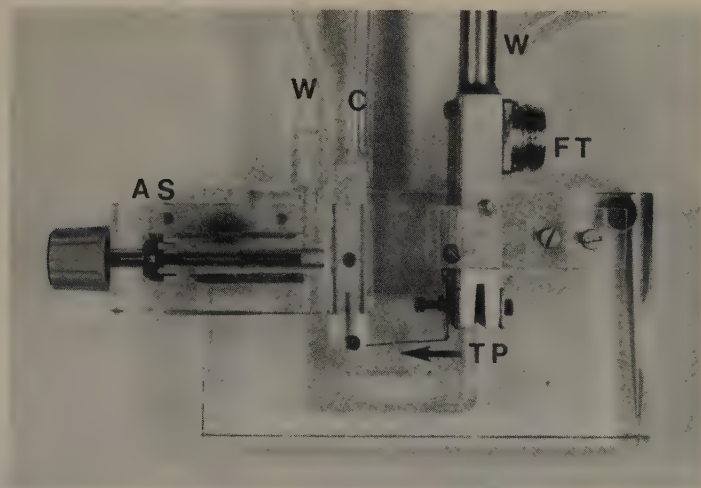


FIGURE 1

Photograph of the 2.5 ml organ bath used in this study. Indicated are tissue prongs (TP), force-displacement transducer (FT), adjustable sled (AS) and tubing for carbogen (C) and circulating 37° C water (W). This device has been described by Högestätt, Andersson & Edvinsson (1983) for studies of mechanical activity in isolated small blood vessels.

The preparations were allowed to equilibrate for 1 to 2 hours and during this time the Krebs solution was changed at 20-minute intervals.

Cumulative concentration-response (C/R) relationships to contractile and relaxant drugs were obtained. Drugs were added to the organ bath in approximately 3-fold increments and responses were allowed to stabilize between additions. When studying contractile agents a maximum concentration of carbachol (10^{-4} M) was always added at the end of each experiment to obtain a defined maximum response.

The preparations did not usually develop tone spontaneously, and, therefore, the bronchioles were contracted by 2×10^{-6} M carbachol (corresponding to 70% of the maximum carbachol contraction) before relaxant responses to bronchodilators were evaluated. In seven rings one or more concentrations of noradrenaline (10^{-7} - 10^{-5} M) were added to the bath before starting other studies.

At the conclusion of each experiment the bronchiolar rings were stored in saline formalin (10% formalin, NaCl 154 mM) for subsequent histological examination. Paraffin-wax embedded specimens were stained with haematoxylin and eosin and, after sectioning to $0.8 \mu\text{m}$, examined by light microscopy. Microscopic examination of stained sections revealed airways of a morphology usually classified as terminal bronchioles - that is a near or total absence of cartilage and the presence of the highly characteristic longitudinally folded mucosa (Bloom & Fawcett 1968, Fig. 2).

For each ring the change in tension in response to each cumulative addition of drug was measured. Generally each specific drug evaluation was made in more than one preparation from the same subject yielding a mean result which was used for the calculations. (Thus, n equals numbers of subjects studied.) C/R curves were constructed by plotting the molar concentration of drug versus contractile or

relaxant response expressed as a percentage of its own maximum response. From this plot the EC50 value (concentration of drug producing 50% of its own maximum effect) was determined. Geometric mean EC50 values were calculated for each substance. To test the significance of differences between geometric mean EC50 values, Student's t-test for non-correlated data was used.

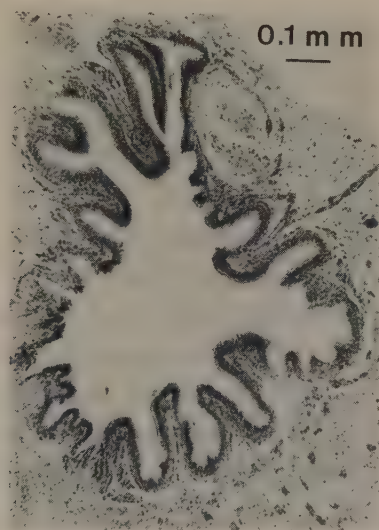


FIGURE 2

Photomicrograph of a cross-section of one of the bronchiolar preparations examined. The bronchiole has a maximum lumen diameter of about 0.6 mm. The preparation was stained with haematoxylin and eosin (see Method).

Solutions of carbamylcholine chloride (Sigma), dipyridamole (Persantine, Boehringer Ingelheim), histamine chloride (Apoteksbolaget) and prostaglandin D₂ (Upjohn) were

prepared daily from a stock solution. Adenosine (Sigma), adenosine 5'-triphosphate (Sigma), substance P (Sigma or Beckman) and (-)noradrenaline bitartrate (Sigma) were dissolved in 154 mM NaCl solution immediately before use and kept on ice (ascorbic acid, 20 µg/ml, was added to the noradrenaline solution). Theophylline (Draco), enprofylline (Draco) and terbutaline (Draco) were diluted from stock i.v. solutions daily.

Results

Bronchiolar rings from eleven patients were studied. The mean age of the patients was 62.5 years (SD 8.8 years) and nine patients were male. All patients had carcinoma of the lung but none had a known history of other airway diseases or airway hyperreactivity. Concentration-response relationships could be obtained from 36 rings. Four preparations exhibited a spontaneous activity of a slow wave-like appearance. These rings were still responsive to drugs but the unstable baseline prevented a quantitative evaluation of drug actions.

Effects of proposed asthma mediators

Carbachol produced a concentration-dependent contraction of the human bronchioles (Fig. 3). Of the mediators studied carbachol produced the largest degree of contraction and at a maximum concentration mean tension was increased 460 ± 135 mg (mean $\pm$ s.e. mean, $n=5$) above the basal tone. The geometric mean EC₅₀ value is shown in Table 1. Responses were stable and reached a plateau 3 to 5 min after addition of each concentration. C/R lines to carbachol were reproducible when repeated 3 to 4 times in each preparation. These carbachol-mediated contractions were completely blocked by pretreatment (15 min) with

atropine ($1\text{ }\mu\text{M}$; $n=3$). Bronchioles contracted dose-dependently also to histamine ($3\cdot 10^{-9}$ - $1\cdot 10^{-4}$ M). The contractions were stable and the maximum contraction obtained was only slightly smaller than that induced by carbachol (Fig 3). The mean EC50 value is shown in Table 1.

Contractile responses were produced by PGD_2 and substance P (Fig 3). PGD_2 was equipotent with carbachol and histamine (Table 1) whereas SP was approximately one order of magnitude less potent (Table 1). PGD_2 produced $68.7 \pm 7.7\%$ ($n=3$) of a maximum carbachol contraction. The C/R curve for SP did not reach its maximum within the limit of the drug's solubility (10^{-4} M).

The purine nucleoside adenosine (10^{-4} M, in the presence or absence of $2\text{ }\mu\text{M}$ of the adenosine uptake-blocking drug dipyridamole) produced only very weak contractions (mean $< 20\%$ of a maximum carbachol contraction, $n=3$) of human bronchioles with a basal tone (Fig 3). The phosphorylated derivative, ATP, induced a larger constriction amounting to about 40% ($n=3$) of that by 10^{-4} M of carbachol (Fig 3). Since only small contractions were produced mean EC50 values were not calculated for the purines. Neither adenosine nor ATP relaxed preparations with a basal tone or contracted by carbachol ($2\cdot 10^{-6}$ M).

Effects of bronchodilators

Preparations with a basal tone could be relaxed by the xanthines but the quantitative C/R relationships were studied only in carbachol-contracted ($2\cdot 10^{-6}$ M, corresponding to 70% of carbachol's maximum) bronchioles. These were concentration-dependently relaxed by theophylline ($1\cdot 10^{-5}$ - $4\cdot 10^{-3}$ M) and enprofylline ($1\cdot 10^{-6}$ - $4\cdot 10^{-4}$ M) (Fig 4). Both xanthines were able to relax the trachea

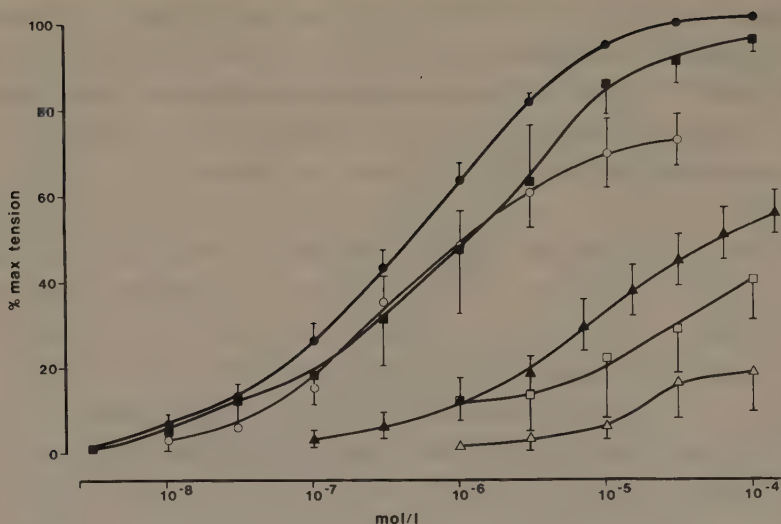


FIGURE 3

Contractile concentration-response curves to carbachol (●, n=5), histamine (■, n=4), prostaglandin D₂ (○, n=3), substance P (▲, n=5), adenosine (△, n=3) and adenosine 5'-triphosphate (□, n=3) obtained in human isolated bronchioles. The substances were added cumulatively and the response is expressed as per cent of a maximum carbachol (10⁻⁴ M) contraction. Mean results are shown and vertical lines indicate s.e.mean.

below the basal tone that existed before the addition of carbachol. EC50 values are shown in Table 1. Enprofylline was found to be approximately 5 times more potent (P<0.01) than theophylline.

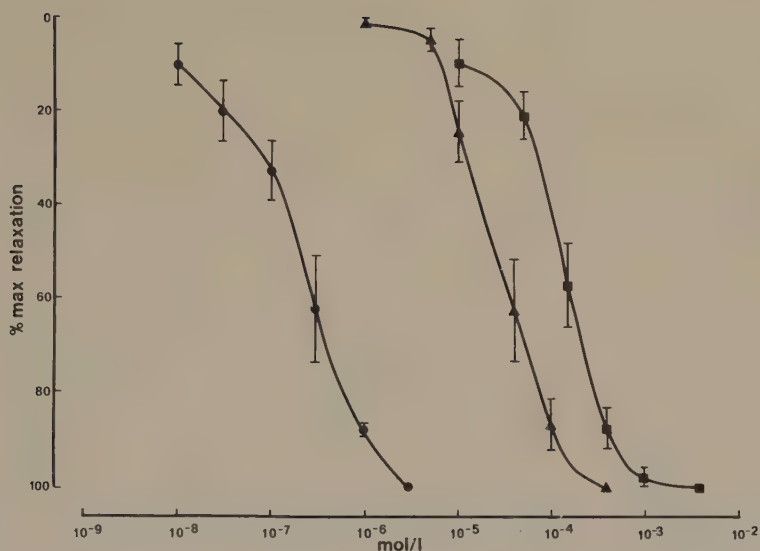


FIGURE 4

Relaxant concentration-response curves to terbutaline (●, n=4), enprofylline (▲, n=6) and theophylline (■, n=5) obtained in human isolated bronchioles contracted by carbachol ($2 \cdot 10^{-6}$ M = EC70). The drugs were added cumulatively and the response is expressed as per cent of its own maximum effect. Mean results are shown and vertical lines indicate s.e.mean.

Relaxant responses to the β_2 -adrenoceptor stimulant terbutaline in carbachol ($2 \cdot 10^{-6}$ M)-contracted preparations were variable. Of eleven preparations studied only four (from four different subjects) responded with concentration-dependent relaxations (Fig 4 and Table 1). Four bronchioles totally failed to relax to terbutaline at

an otherwise almost maximally effective concentration (10^{-6} M), yet theophylline and enprofylline relaxed these rings completely. C/R curves of the xanthines in terbutaline- unresponsive preparations showed no significant changes. The remaining three rings responded to terbutaline in degrees between these two extremes.

Table 1

Bronchoconstrictors	- log EC50	n
Carbachol	6.40 ± 0.09	5
Histamine	6.20 ± 0.29	4
Prostaglandin D ₂	6.39 ± 0.12	3
Substance P	5.34 ± 0.20	5
Bronchodilators		
Theophylline	3.90 ± 0.08	5
Enprofylline	4.59 ± 0.13	6
Terbutaline	6.70 ± 0.13	4

n equals number of subjects studied

Responses to one or more concentrations of noradrenaline ($1 \cdot 10^{-7}$ - $1 \cdot 10^{-5}$ M) were examined in seven preparations with a basal tone. At these concentrations, noradrenaline always produced some degree of relaxation. Only this qualitative aspect of noradrenaline was evaluated and noradrenaline was not examined in the presence of carbachol.

Discussion

This study showed that isolated bronchioles from human lung responded in a sensitive and reproducible way to drugs and asthma mediators. Thus constrictory agents produced stable contractions which could be relaxed by bronchodilator drugs. In contrast to the lung parenchymal strip preparations, where many types of contractile tissues are involved, these isolated bronchioles should, indeed, reflect small airway smooth muscle reactivity. Upon stimulation with mediators the bronchioles were able to generate large degrees of tension. The tension changes were immediate and reversible, which is compatible with the view that peripheral airways narrowing in lung disease may in part be due to rapid changes in the contractile state of the smooth muscle (McFadden & Ingram 1979).

We examined effects upon bronchiolar tone of some established or putative neurotransmitters that may play a role as asthma mediators. The adrenergic neurotransmitter noradrenaline frequently contracts, via α -receptor stimulation, the human lung parenchymal strip preparation (Black, Turner & Shaw 1981, Goldie, Paterson & Wale 1982, Bertram et al 1983), and in small airways of ferrets relatively large numbers of α -adrenoceptors are present (Barnes, Basbaum, Nadel & Roberts 1983). It has also been shown that α -adrenoceptor blocking drugs may possess some anti-asthmatic actions (Dyson, Hills, Mackay & Wood 1980, Barnes, Wilson & Vickers 1981, Marlin, Thomson, Chow, Reddell & Cheng 1981). However, the isolated bronchioles responded only with relaxation to noradrenaline. This observation does not support a role for this amine in small airways constriction. Rather, it is likely that tissues other than small airways, as for example vascular smooth muscle, mediated the contractile response to

noradrenaline in lung strip preparations (Goldie et al 1982, Bertram et al 1983). Hence, a relaxant effect or lack of contraction by noradrenaline may demonstrate that the preparation under study is a small airway and not a blood vessel.

Human peripheral airways and alveolar ducts are cholinergically innervated (Larsell & Dow 1933, Gaylor 1934) and it cannot be excluded that the bronchodilator activity of atropine-like drugs in asthmatic subjects (Simonsson 1977) includes effects on small airways. To mimic the effects produced by the parasympathetic neurotransmitter acetylcholine we employed the drug carbachol. It was found to be a potent constrictor of the human bronchioles. It acted via muscarinic receptors, since the contractions were blocked by atropine. The present observations support the view that cholinergic constriction of small airways is of importance.

Nerve fibres with immunoreactivity to the smooth muscle constrictory peptide substance P are present in human airways (Lundberg, Hökfelt, Martling, Saria & Cuello 1984). Substance P contracted human bronchioles, but it was about one order of magnitude less potent than carbachol and its maximum effect was small. It cannot be excluded that substance P or some other, more potent, bronchoconstrictory tachykinin peptide (Karlsson, Finney, Persson & Post 1984) participates in small airways neural constriction.

The lung mast cell is generally recognized as a major source of non-neurally derived putative asthma mediators (see Lagunoff 1983). PGD₂ is the quantitatively predominant cyclooxygenase product released from sensitized human lung tissue (probably from mast cells) after challenge in

vitro with antigen (Schulman, Newball, Demers, Fitzpatrick & Adkinson 1981). The present study has demonstrated that it is almost as effective as the traditional asthma mediator, histamine, in contracting human small airways. Also, inhaled PGD_2 has recently been shown to be a potent bronchoconstrictor in man (Hardy, Robinson, Tattersfield & Holgate 1984). Thus PGD_2 may well be as important as histamine for small airways constriction in obstructive airways diseases. The purine compounds adenosine and ATP have been suggested to be released from neural or non-neural tissues and to modulate airway tone (Fr  dholm, Brodin & Strandberg 1979, Cushley, Tattersfield & Holgate 1983, 1984). Given by inhalation, both adenosine and ATP produced a marked bronchoconstriction in asthmatic subjects (Cushley et al 1983, 1984). In the present study adenosine produced only weak and inconsistent contractions whereas ATP was a slightly more effective constrictor. In larger human bronchi (> 2 mm) adenosine ($\leq 3 \cdot 10^{-4}\text{M}$) was completely without effects on tone (unpublished observations). In animal studies, adenosine had variable but predominantly relaxatory responses (Coleman 1976, Farmer & Farrar 1976, Karlsson, Kjellin & Persson 1982) but ATP produced bronchoconstriction (Farmer & Farrar 1976, Lundblad, Karlsson & Persson 1984). Since the antiasthmatic drug theophylline is a potent adenosine antagonist, the possibility that adenosine is an asthma mediator has received much interest. The poor response found in this study suggests that this purine is not important in small airways contraction.

Our findings thus do not support the view that noradrenaline or adenosine contributes to bronchiolar constriction in asthma because relaxant effects and very weak contractions, respectively, were produced. Naturally, it can be argued that bronchiolar smooth muscle from asthmatics

might have responded in a different way than our preparations which were obtained from patients with lung carcinoma and without asthma. This argument may not be altogether valid, since in studies of contractile mediators it has not been possible to separate airways reactivity in isolated bronchi (and parenchymal strips) from normals and asthmatics (Vincenc, Black, Yan, Armour, Donnelly & Woolcock 1983, Finney et al 1983b, Roberts, Raeburn, Rodgers & Thomson 1984). On the other hand, the present results support the possibility that histamine, PGD_2 , substance P, ATP, and acetylcholine (mimicked by carbachol) participate in bronchiolar constriction in asthma. Although these agents are known to be present in the lung, their roles in asthma are difficult to assess since the relevant concentrations of these agents at target cells like the bronchiolar smooth muscle cell are not known.

It is of interest for the evaluation of in vitro findings that drugs with acute and significant effects on asthma symptoms are known to be active in patients within certain plasma (and tissue) levels. Systemic treatment with β -adrenoceptor stimulants like terbutaline and salbutamol results in bronchodilation at 10^{-8} - 10^{-7} M in plasma (e.g. van den Berg, Leferink, Maes, Fokkens, Kreukniet & Bruynzel 1984). However, it was reported that 10^{-7} - 10^{-3} M of salbutamol was needed to relax isolated human bronchi with a basal tone and that salbutamol was almost without effect in histamine-contracted preparations ($\text{EC}_{50}=10^{-3}$ M) (Davis, Conolly & Greenacre 1980). We were particularly interested in the possibility suggested by Persson & Ekman (1976) that human bronchiolar strips could respond to low concentrations of a β -adrenoceptor stimulant, and we considered those preparations unresponsive that did not relax at 10^{-6} M of terbutaline. Four out of eleven car-

bachol-contracted bronchioles were sensitive to terbutaline suggesting that β_2 -adrenoceptor stimulants are potent relaxants of small airways at least in a fraction of human subjects. It is conceivable that large concentrations, such that would result from high doses of terbutaline given by the inhaled route, are needed to produce relaxation of bronchiolar muscle in many patients.

In contrast to the effect of β_2 -adrenoceptor stimulation the bronchiolar relaxation induced by xanthines was consistent, and a significant portion of the response was obtained within clinically relevant concentrations of theophylline and enprofylline. The finding that xanthines also relaxed preparations poorly responsive to terbutaline suggests that the inability of this agent to relax bronchiolar rings was due to some limitation in the β_2 -adrenoceptor function rather than to relaxant properties of the isolated bronchiolar smooth muscle. The dissimilar relaxant responses are surprising as both β_2 -receptor agonists and xanthines relax airway smooth muscle through functional antagonism, i.e. irrespective of which type of asthma mediator that has produced constriction (Finney, Gustafsson, Karlsson, Persson & Sonmark 1983a). However, the elevated tension (induced by carbachol) per se could have contributed to the difference since xanthines, but not β -receptor agonists, readily relax highly contracted guinea-pig tracheal muscle (Karlsson & Persson 1981).

Enprofylline has been characterized as an adenosine non-blocking xanthine whereas theophylline is an effective antagonist of the effects produced by adenosine. The observation that enprofylline was more potent than theophylline as a human bronchiole relaxant in vitro agrees with the bronchodilator potencies observed in human large airways in vitro (Persson et al 1981) and in asthmatic

subjects in vivo (e.g. Lunell, Svedmyr, Andersson & Persson 1982). It also gives further support to the view that adenosine antagonism does not explain the antiasthmatic lung actions of xanthine derivatives (Persson, Erjefält, Edholm, Karlsson & Lamm 1982, Persson 1984). Both xanthines are able to inhibit human cyclic AMP phosphodiesterase enzymes, enprofylline being about twice as potent as theophylline (unpublished observations by Bergstrand, see Bergstrand 1980). However, large concentrations of the xanthines are needed for phosphodiesterase inhibition (see Bergstrand 1980), and xanthine derivatives have relaxant characteristics which are difficult to reconcile with a cyclic AMP-mediated response (Kolbeck, Speir, Carrier & Bransome 1979, Karlsson & Persson 1981). Notwithstanding the fact that the subcellular mechanisms involved remain unsettled, xanthines like theophylline and enprofylline have potent small airways relaxant effects, which are suggested to be of therapeutic importance.

In conclusion, it is suggested that the present method of using bronchioles in vitro, in contrast to the lung parenchymal strip technique, is suitable for the study of human small airways reactivity. A number of different mediators (substance P, carbachol, ATP, histamine, PGD_2) of neural, mast cell, or other origin were found to be constrictors of the human bronchioles. Observations with noradrenaline and adenosine did not support a role for these agents in small airways constriction. Xanthines consistently, and a β_2 -adrenoceptor stimulant less consistently, relaxed the human bronchioles at concentrations considered to be clinically relevant. The adenosine non-blocking xanthine enprofylline was a potent relaxant supporting the view that the antiasthmatic lung actions of xanthines are not due to adenosine receptor antagonism.

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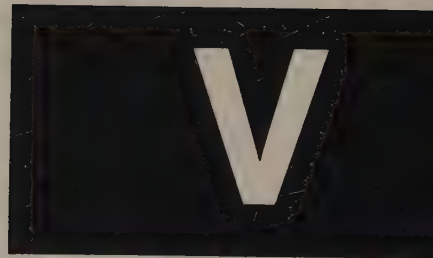
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NEITHER VASOACTIVE INTESTINAL PEPTIDE (VIP) NOR
PURINE DERIVATIVES MAY MEDIATE NON-ADRENERGIC
TRACHEAL INHIBITION

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Abstract

When guinea-pig tracheas are stimulated in an isolated electrical field, the neurogenic responses include a prominent relaxation that is only partially inhibited by propranolol. The possibility that either purine derivatives or VIP could be a non-adrenergic inhibitory neurotransmitter was examined. Relaxant characteristics of adenosine, ATP, adenine and VIP in tracheas with different degrees of contraction were compared to those of maximum non-adrenergic nerve stimulation. Spontaneously- and carbachol (5.5 μ M and 55 μ M)-contracted tracheas were inhibited $77.5 \pm 5.0\%$ (n=9), $24.7 \pm 2.8\%$ (n=11) and $14.5 \pm 5.0\%$ (n=10), respectively, by electrical nerve stimulation. In these three states of contraction exogenous VIP, but not adenosine, ATP or adenine, produced relaxations similar to those of electrical nerve stimulation. In carbachol-contracted tracheas with maximally effective concentrations of adenosine (3 mM), adenine (3 mM) or VIP (23.3 μ M) present, the non-adrenergic neurogenic inhibitory response remained intact. The neurogenic inhibitory response was also unaffected by the presence of theophylline and indomethacin which

blocked relaxatory effects of adenosine and ATP, respectively. The results suggest that neither the purine derivatives, nor VIP are involved in the non-adrenergic, neurogenic inhibition in the guinea-pig trachea.

Introduction

When guinea-pig tracheas are stimulated in an isolated electrical field, the neurogenic responses include a prominent smooth muscle relaxation. The inhibitory response is not at all or only slightly antagonised by propranolol, indicating that the adrenergic component is minor. Non-adrenergic (non-cholinergic) inhibition is thus reported to be the dominant or exclusive neural relaxatory response in guinea-pig, cat, baboon, bovine, and human isolated lower airways (Coburn & Tomita 1973, Richardson & Beland 1976, Chesrown et al 1980, Diamond & O'Donnell 1980, Middendorf & Russel 1980, Yip et al 1981, Ito & Takeda 1982, Cameron et al 1983). Identification of the non-adrenergic neurotransmitter should be of importance for our understanding of neural regulation of bronchial tone. Furthermore, a dysfunction of the inhibitory nervous system has been proposed to be significantly involved in diseases such as bronchial asthma (e.g. Richardson & Bouchard 1975).

The non-adrenergic inhibition has been suggested to be mediated by a purine derivative (Coleman & Levy 1974, Souhrada et al 1980, Satchell 1982) but arguments against this view have been raised (Kamikawa & Shimo 1976b, Ito & Takeda 1982, Irvin et al 1982, Cameron et al 1983). Another apparently strong candidate for the mediation of non-adrenergic inhibition is the airway relaxant, vaso-

active intestinal peptide (VIP), whose presence in nervous structures within the airway has been shown with immunohistochemical techniques (Uddman et al 1978, Håkanson et al 1983). As a putative neurotransmitter in airways, VIP has already been tried as an antiasthmatic agent (Bundgaard et al 1983, Morice et al 1983, Barnes et al 1984). In both cat and bovine tracheal preparations very similar effects were produced both by exogenous VIP and non-adrenergic inhibitory nerve stimulation on the electrical and mechanical properties of the smooth muscle (Ito & Takeda 1982, Cameron et al 1983). Perhaps the most intriguing evidence favouring VIP as the non-adrenergic inhibitory neurotransmitter was obtained in a study of guinea-pig tracheal preparations: Matsuzaki et al (1980) reported that a VIP-like substance (radioimmunoassay) was released during electrical field stimulation. The release and the neurogenic inhibition were correlated and both were abolished by pretreatment with tetrodotoxin (TTX). Moreover, prior incubation of the trachea with antiserum to VIP reduced the relaxatory response to electrical field stimulation (Matsuzaki et al 1980). However, these workers obtained surprisingly small tracheal relaxatory responses to an intense nerve stimulation and their experiments with antiserum to VIP were difficult to interpret, partly because they expressed the induced antagonism in percentage of a poorly defined relaxant response (electrically induced, TTX-resistant relaxations).

By examining drug interactions in tracheal muscle pre-contracted to various degrees, we have previously been able to distinguish qualitatively between effects produced by different relaxatory agents (Karlsson & Persson 1981). In order to study further the nature of the non-adrenergic inhibitory neurotransmitter, effects of exogenously added purine derivatives and VIP, and their interactions with

non-adrenergic nerve stimulation have now been examined in contracted guinea-pig tracheas. Part of these results have been reported elsewhere (Karlsson & Persson 1983).

Method

Guinea-pigs of either sex (Dunkin-Hartley, body-weight 300-600 g) were killed by a blow to the head. The trachea was rapidly dissected out and placed in Krebs solution (composition in mM: NaCl 118.0, KCl 4.6, CaCl_2 2.5, MgSO_4 1.15, NaHCO_3 24.9, KH_2PO_4 1.15, glucose 5.5 with a pH of 7.4). Connective tissue was carefully trimmed away from the trachea. Two adjoining rings from the midportion of the extra-thoracic trachea were cut out in one piece. At least two such preparations were obtained from each animal. The intact ring was thread on two metal prongs that were placed in a 2.5 ml organ bath with heated (37°C) Krebs solution (gassed with 5% CO_2 in oxygen).

One metal prong was firmly attached to an adjustable sled and the other was connected to a strain-gauge transducer (Grass FT03). Isometric tension-changes were measured and recorded on a Grass Polygraph model 5. The preparations were given an initial tension of 0.6 g (6 mN) by adjusting the sled. The rings were allowed to equilibrate for at least one hour during which time the preparations were repeatedly washed. During this time period the tension usually increased spontaneously.

The rings were electrically stimulated by use of a Grass S88 stimulator. The prong attached to the sled and a platinum pin placed on the opposite side of the other prong were used as electrodes. The distance between them was 3-5 mm. TTX ($3.1 \mu\text{M}$) was used to ascertain that only nerves were being stimulated (Kao, 1966).

The drugs were added by injection to the organ bath, either as single doses or in a cumulative progression of concentrations until no further effect was obtained with the next higher concentration. The total added volume during one experiment did not exceed 200 μ l.

Relaxant responses to field stimulation or drugs were calculated as a percentage of the tone that existed immediately before electrical stimulation was started or the drugs were added to the organ bath. Mean $\pm$ SE values are reported in the Results section and are calculated from data obtained from at least four different animals. The EC50 value is defined as the concentration of drug that produced 50% of its own maximum response. Statistical evaluations were made by use of the Student's t-test for unpaired observations.

Drugs used: Adenine dihydrochloride (Sigma), adenosine (Sigma), adenosine 5'-triphosphate disodium (ATP; Sigma), atropine sulphate (Apoteksbolaget), carbachol chloride (Ph Nord), dipyridamole (Persantine, Boeringer-Ingelheim), enprofylline, anhydrous (Draco), isoprenaline hydrochloride (Sigma), propranolol dihydrochloride (Hässle), tetrodotoxin (TTX, Sankyo), theophylline, anhydrous (Knoll), vasoactive intestinal peptide, porcine (VIP; V. Mutt).

The xanthines were dissolved in 1 equiv. 0.5 M NaOH and diluted with Krebs solution. All other drugs were both dissolved in and diluted with Krebs solution.

Results

Neurogenic effects on tone in guinea-pig isolated tracheas.

Electrical nerve stimulation of tracheas with an intrinsic tone produced a biphasic response. This consisted of an initial contraction immediately followed by a pronounced relaxation, after which the preparation spontaneously regained tone (Fig 1). Repeated stimulation of tracheas with intrinsic tone produced contractions with a gradually diminishing amplitude. The neurogenic contractions were completely abolished by pretreatment with atropine ($1\text{ }\mu\text{M}$, 15 min) (Fig. 1). When the tracheal tone had been enhanced by $5.5\text{ }\mu\text{M}$ or $55\text{ }\mu\text{M}$ carbachol (atropine-sensitive contractions, corresponding to 95% and 100% of a maximum carbachol contraction, respectively; see Karlsson & Persson 1981) the atropine-sensitive neurogenic contraction was abolished and only the inhibitory response remained (Fig. 4).

To obtain maximum inhibitory neural responses the preparations were electrically stimulated (in the presence of $0.1\text{ }\mu\text{M}$ propranolol) with trains of pulses for 2 min (pulse duration 0.5 ms, frequency 20 Hz) at a supramaximal voltage. The stimulation parameters were almost identical but we obtained much larger non-adrenergic inhibitory responses in the guinea pig trachea than did Matsuzaki et al (1980). Stimulation for longer periods of time produced a slightly diminished response. After a stimulation period of 12 min (in the presence of $5.5\text{ }\mu\text{M}$ carbachol) $81.8 \pm 9.0\%$ ($n=4$) of the initial maximum inhibition remained. Stimulation with a frequency of 50 Hz did not produce significant additional inhibition compared with 20 Hz. With a longer pulse duration ($> 0.5\text{ ms}$) the responses were

not completely blocked by the prior (15 min) addition to the bath of TTX ($3.1 \mu\text{M}$), indicating that with longer pulse durations non-nervous mechanisms were activated.

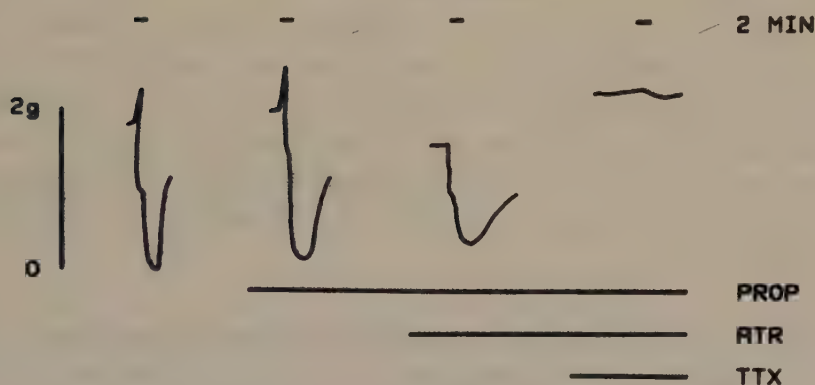
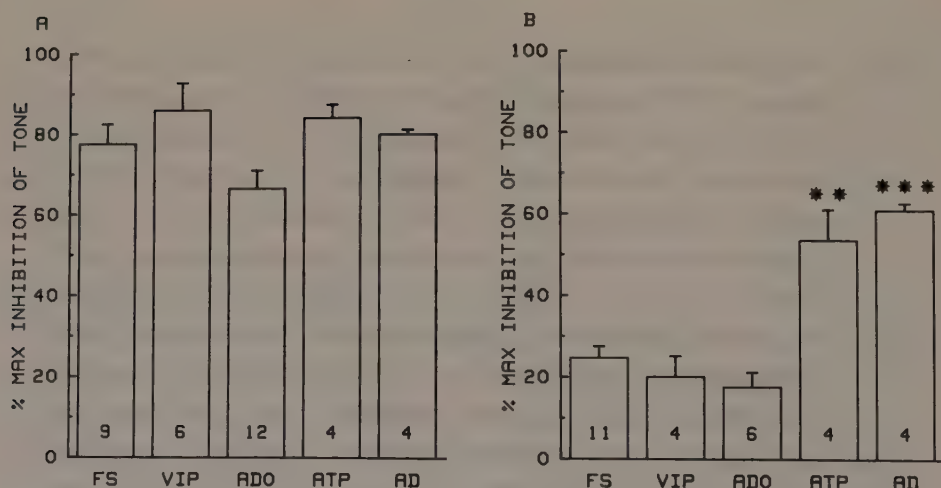


FIGURE 1

Original tracings from electrical field stimulated (FS; trains of pulses with a frequency of 20 Hz and 0.5 ms duration, at supramaximal voltage) guinea-pig tracheal ring preparations with a spontaneous tone. Periods of FS are indicated by horizontal lines above tracings. To the left is shown an electrically induced biphasic response, including a pronounced off relaxation (see Results). The biphasic response is unchanged in the presence of propranolol (Prop, $0.1 \mu\text{M}$). Atropine (Atr, $1 \mu\text{M}$) blocks the FS-induced contraction and the remaining non-adrenergic inhibitory response is abolished by tetrodotoxin (TTX, $3.1 \mu\text{M}$).

The neural inhibitory response began during electrical stimulation and continued after the stimulation had been stopped. This relaxatory response has accordingly been divided into on- and off-relaxations, respectively (Figs. 1 and 4, see Daniel et al 1980, Cameron et al 1983). In preparations with an intrinsic tone the amplitude of the off-relaxation corresponded to $21.1 \pm 7.6\%$ (range: 0-62.8%; n=9) of the total relaxatory peak response (on-plus off-relaxation). The relative magnitude of the off-relaxation was unchanged ($19.0 \pm 3.2\%$, n=8) in carbachol contracted preparations ($5.5 \mu\text{M}$). Atropine ($1 \mu\text{M}$) treatment of preparations with intrinsic tone (n=5) changed the inhibitory response to nerve stimulation to only on-relaxation (Fig. 1).

Within 10-15 min after the electrical stimulation, the tone had returned approximately to the pre-stimulation



FIGURES 2A AND 2B

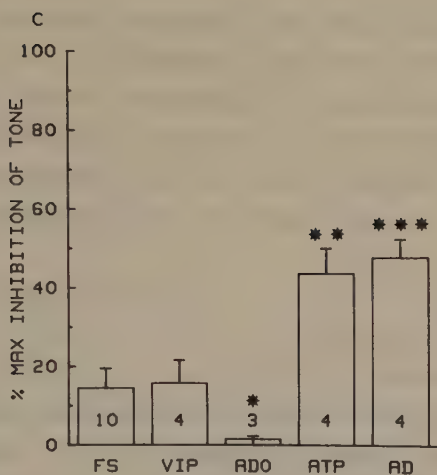


FIGURE 2C

Maximum relaxant responses to field stimulation (FS, see Fig. 1; 0.1 μ M propranolol present), vasoactive intestinal peptide (VIP; 1.3 μ M in spontaneously contracted and 23.3 μ M in carbachol contracted rings), adenosine (ADO; 3 mM in the presence of 2 μ M dipyridamole), ATP (3 mM) and adenine (AD, 3 mM) obtained in guinea-pig tracheal preparations with either a spontaneous tone (2A) or contracted by 5.5 μ M (2B) or 55 μ M carbachol (2C).

The bars illustrate per cent maximum inhibition of tracheal tone. Vertical lines represent SE and numbers within the bars indicate number of preparations studied. The statistical difference between the maximum effect of FS and either compound is indicated above the bars (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$; Student's t-test).

level. During this recovery time the tracheas were either completely refractory to electrical stimulation or responded with a much diminished relaxation. With sufficiently long time intervals (> 20 min) between stimulations the inhibitory response was intact when stimulations were repeated 3 to 4 times in individual preparations.

Tracheal tone and neural relaxation

Electrical nerve stimulation for 2 min (pulse duration 0.5 ms, frequency 20 Hz, supramaximal voltage; 0.1 μ M propranolol present) inhibited about 80% of the tone that existed in spontaneously contracted preparations (Figs. 1 and 2). In carbachol-contracted tracheas the inhibitory effect was reduced. With 5.5 μ M and 55 μ M carbachol present, field stimulation reduced the tone by about 25% and 15%, respectively (Figs. 2 b and c). Maximum neurogenic responses, whether in the absence or presence of carbachol, were not affected by pretreatment for 30 min with either 3.1 μ M indomethacin or 0.1 μ M propranolol (a concentration that completely blocked the relaxant effect of a maximally effective concentration of isoprenaline, 1.0 μ M; data not shown).

Effects of adenosine

Adenosine relaxed the tracheal rings (dipyridamole; 2 μ M, was present to block the cellular uptake of adenosine). The degree of relaxation to adenosine diminished markedly with increasing concentration of carbachol (Fig. 2; see also Karlsson et al 1982).

When the tracheas had been contracted by 55 μ M carbachol, adenosine, at maximally soluble concentrations (3.0 mM),

was almost inactive, inhibiting less than 3% of the tone (Fig. 2c). Adenosine always caused considerably smaller relaxations than did either electrical nerve stimulation or VIP (see below). Neither propranolol (0.1 μ M), nor indomethacin (3.1 μ M) affected the adenosine-induced relaxation.

Effects of ATP

ATP (3×10^{-5} - 3×10^{-3} M) relaxed both tracheas with an intrinsic tone and those previously contracted by carbachol. The relaxant response was not stable, therefore, the nadir was measured. Low concentrations of ATP have been shown to transiently contract tracheas with a medium tone (Kamikawa & Shimo 1976a, Lundblad et al, 1984). Such a contraction to ATP was not found in this study, probably because the preparations had spontaneously gained a high tone (1.7 ± 0.3 g, $n=4$). Spontaneously contracted tracheas were almost completely relaxed by ATP ($83.9 \pm 3.9\%$, $n=4$ Fig. 2a). The degree of relaxation diminished with an increase in carbachol concentration (Figs. 2 and 3a). At a maximum carbachol concentration (55 μ M), ATP, at maximally soluble concentrations (3.0 mM), inhibited the tone by $43.6 \pm 6.4\%$ ($n=4$). With either 5.5 μ M or 55 μ M carbachol present, exogenous ATP produced significantly ($P < 0.01$) larger relaxations than did maximum non-adrenergic, inhibitory nerve stimulation (Fig. 2). ATP-induced relaxations were unaffected by propranolol (0.1 μ M) but were reduced by $> 90\%$ after pretreatment with indomethacin (3.1 μ M, $n=4$, Lundblad et al 1984). Indomethacin was without effect on the non-adrenergic inhibition.

Effects of adenine

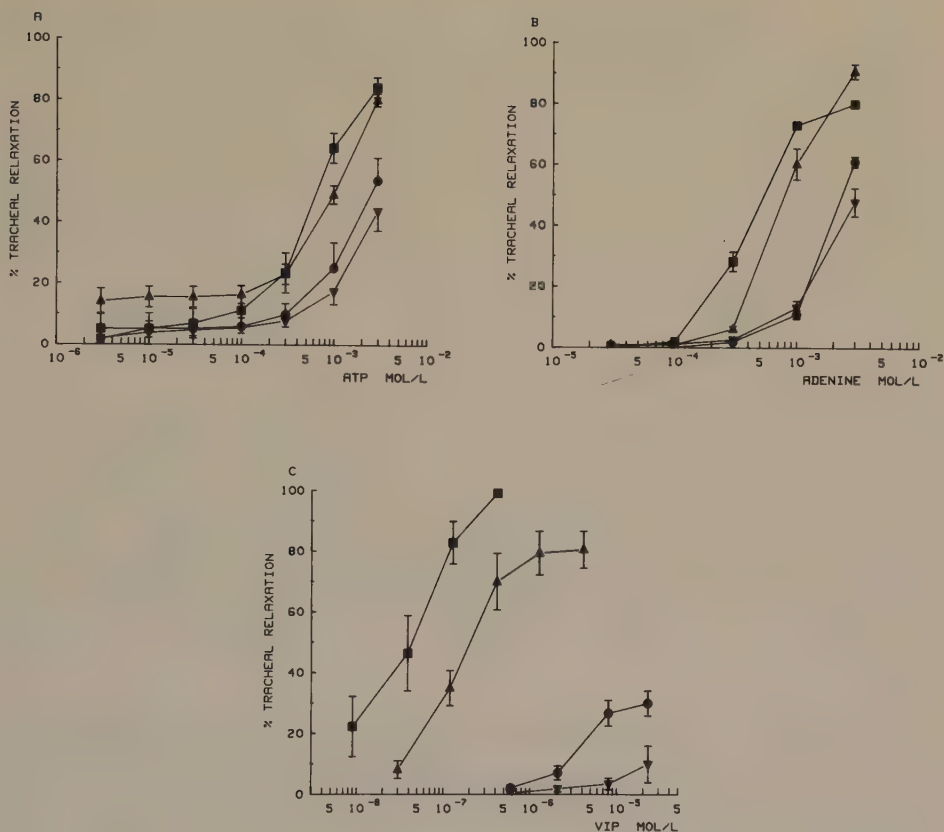
Adenine (3×10^{-5} - 3×10^{-3} M) produced concentration-related relaxations. Tracheas with a low tone could be almost completely relaxed (intrinsic tone: by $79.7 \pm 1.2\%$, $n=4$ Fig. 2b). The adenine actions were reduced by the presence of carbachol, but were still larger than the relaxant responses to inhibitory nerve stimulation (Figs. 2 and 3). The effect of adenine remained intact in the presence of propranolol ($0.1 \mu\text{M}$) and indomethacin ($3.1 \mu\text{M}$).

Effects of VIP

VIP relaxed $85.9 \pm 6.8 \%$ ($n=6$) of the intrinsic tracheal tone (Fig. 2 a). With increasing concentrations of carbachol, both potency and efficacy of VIP were diminished (Figs. 2b, 2c and 3c). The mean degrees of relaxation for VIP were always of the same magnitude as those for electrical nerve stimulation (Fig 2). Individual preparations, could, however, respond with a neural relaxation that differed from that produced by VIP (see Fig 6)(Fig. 4). VIP-induced relaxations were unchanged by pretreatment with either $0.1 \mu\text{M}$ propranolol or $3.1 \mu\text{M}$ indomethacin.

Interactions between neural inhibition and putative neurotransmitters

Since tracheas with a low tone could be almost completely relaxed by either ATP, adenine, VIP or non-adrenergic inhibitory nerve stimulation (Fig. 2a), interactions between the inhibitory neural response and these agents had to be evaluated in carbachol-contracted ($5.5 \mu\text{M}$ or $55 \mu\text{M}$) tracheas ($0.1 \mu\text{M}$ propranolol was present throughout these experiments). After addition of carbachol the preparations were electrically stimulated for 2 min to



FIGURES 3A, 3B AND 3C

Tracheal relaxant effects of ATP, adenine and vasoactive intestinal peptide (VIP). The compounds were added cumulatively to guinea-pig isolated tracheas with a spontaneous tone (■) or contracted by three different concentrations of carbachol (0.55 μ M, ▲; 5.5 μ M, ●; and 55 μ M, ▼). The relaxation is expressed as per cent inhibition of tracheal tone. Each curve represents mean results in tracheas obtained from 4-6 animals. Vertical lines indicate SE.

obtain a control response in each preparation. When the tracheas had regained tone, concentration-response relationships to the purines or VIP were evaluated. In the presence of maximum concentrations of adenosine, adenine or VIP the effect of a second period of electrical stimulation (2 min) was evaluated. ATP-induced relaxations were not stable, invalidating an interaction study between this purine and the neurogenic response.

The maximum non-adrenergic neural inhibition (at 5.5 μ M carbachol) was apparently not affected by the presence of maximal concentrations of either adenosine or VIP (Figs. 4 and 5). In preparations contracted by 55 μ M carbachol, the maximum inhibitory response to electrical nerve stimulation was not reduced in the presence of maximum concentrations of either adenosine, VIP, or adenine (Figs. 4 and 5).

In addition, the response of VIP was found to be unaffected by a maximum neurogenic inhibition. VIP (6.0 μ M) was added 2 to 3 min after the start of a prolonged (12 min) electrical stimulation. The mean relaxation to VIP in the presence of electrical stimulation ($18.7 \pm 3.2\%$, $n=5$) was not significantly different ($P > 0.05$, Student's t-test) from that obtained in the absence of electrical stimulation ($19.1 \pm 6.5\%$, Fig. 6). It was then examined, whether a decrease in tone by itself would affect the magnitude of the non-adrenergic inhibitory neural response. Carbachol-contracted (5.5 μ M) rings were relaxed by 0.3 - 1.0 mM theophylline to a degree comparable with that produced by maximum concentrations of VIP. In the presence of these concentrations of theophylline, where the adenosine antagonistic property of this drug would be fully expressed (see Karlsson et al 1982), the neurogenic inhibition of tone was not significantly different from

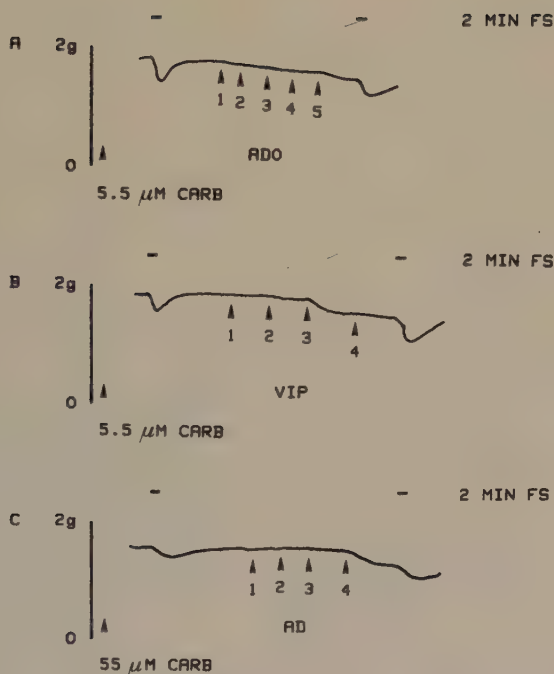


FIGURE 4

Original tracings from carbachol-contracted guinea-pig tracheal rings. Electrical field stimulation (FS, see Fig. 1; $0.1 \mu\text{M}$ propranolol present) for 2 min (horizontal lines above tracings) produces a relaxant effect that is not changed by the presence of maximum concentrations of adenosine (A; ADO, 1= 0.03 mM , 2= 0.1 mM , 3= 0.3 mM , 4= 1.0 mM , 5= 3.0 mM), vasoactive intestinal peptide (B; VIP, 1= $0.8 \mu\text{M}$, 2= $1.5 \mu\text{M}$, 3= $6.0 \mu\text{M}$, 4= $15.0 \mu\text{M}$) or adenine (C; AD, 1= 0.1 mM , 2= 0.3 mM , 3= 1.0 mM , 4= 3.0 mM).

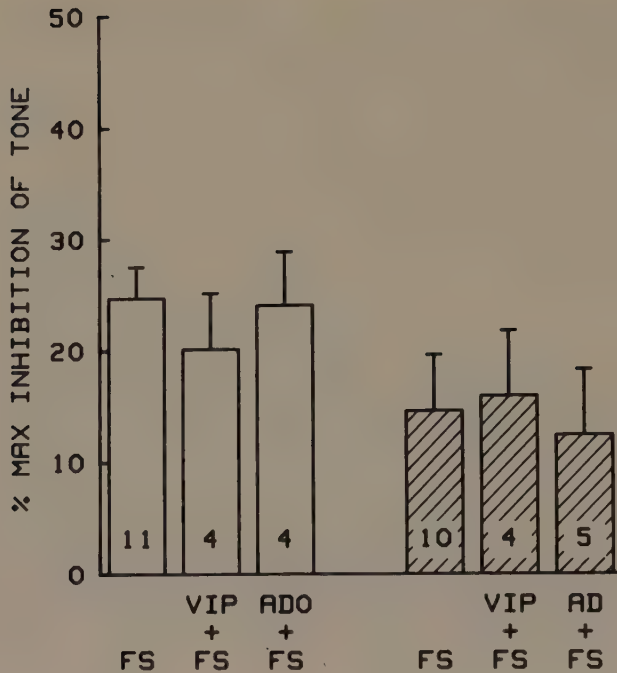


FIGURE 5

Neural inhibition of tracheal tone in the absence and presence of maximum concentrations of vasoactive intestinal peptide (VIP; 23.3 μ M), adenosine (ADO, 3 mM) or adenine (AD, 3 mM). Tracheas contracted by 5.5 μ M ($\square$) or 55 μ M ($\text{\textbackslash}$) carbachol were electrically stimulated for 2 min (FS, see Fig. 1, 0.1 μ M propranolol present). Each bar represents mean values from 4 to 11 animals. Vertical lines indicate SE. The magnitude of the neural inhibitory response in the absence of drugs was not significantly different ($P > 0.05$, Student's t-test) from the inhibition in the presence of either VIP, ADO or AD.

Table 1. Non-adrenergic neural inhibition of tracheal tone is not affected by the presence of relaxant concentrations of xanthines. Guinea-pig tracheal rings, contracted by 5.5 μ M carbachol were relaxed about 25% by 0.2-1.0 mM theophylline (THEO) or by 0.05-0.1 mM enprofylline (ENPRO). The magnitude of the inhibitory response to a 2 min electrical field stimulation (stimulation frequency 20 Hz, pulse duration 0.5 ms, supramaximal voltage; 0.1 μ M propranolol present) was evaluated in the absence and presence of THEO and ENPRO. Mean $\pm$ SE values are shown.

	Tracheal tone before stim mg	Neural inhibition of tracheal tone mg	%	n ^a
Stim. in absence of THEO	2360 $\pm$ 500	530 $\pm$ 163	21.8 $\pm$ 3.3	4
Stim. in presence of THEO	1620 $\pm$ 330	477 $\pm$ 51	31.8 $\pm$ 4.9	4
Stim. in absence of ENPRO	3200 $\pm$ 370	506 $\pm$ 125	15.6 $\pm$ 2.2	5
Stim. in presence of ENPRO	2160 $\pm$ 250	540 $\pm$ 124	23.0 $\pm$ 3.9	4
				NS ^b
				NS

a) Number of animals

b) Not significant ($P > 0.05$, Student's t-test)

that obtained in either untreated or VIP-relaxed preparations ($P > 0.05$, Student's t-test, Table 1). Similar results were obtained when the tone had been reduced by enprofylline (0.05 - 0.1 mM, Table 1), a xanthine derivative with a negligible ability to antagonise adenosine and with a relaxant potency about 5 times that of theophylline (Persson et al 1982). Contrary to what would be expected if adenosine is the neurotransmitter, the neural response seemed to be slightly enhanced in the presence of theophylline. This augmentation may simply be due to a reduced tracheal tone compared with the controls since both xanthines shared this action (Table 1). Finally, it was observed that relaxatory responses to theophylline (1.0 mM) and enprofylline (0.24 mM) obtained during inhibitory nerve stimulation (12 min) did not differ significantly ($P > 0.05$, Student's t-test; Table 2) from their respective control values. Accordingly, the comparison made between relaxant responses to VIP during field stimulation and control conditions should be valid.

Discussion

In the presence of a muscarinic receptor antagonist, electrical field stimulation of guinea-pig isolated tracheas induced a pronounced relaxatory response that was propranolol-resistant. Prostaglandins are probably not involved in this response since it was also resistant to indomethacin treatment. This non-adrenergic neurogenic inhibition was fully developed by electrical stimulation for 2 minutes. In agreement with previous studies (Kamikawa & Shimo 1976 b, Kalenberg & Satchell 1979, and Souhrada et al 1980) the degree of activation of these nerves was frequency-dependent with a maximum stimulation occurring at high frequency (≥ 20 Hz). In highly contracted tracheas the non-adrenergic inhibitory effect was

Table 2. Relaxant effects of xanthines in guinea-pig tracheas are not affected by maximum non-adrenergic inhibitory nerve stimulation. In carbachol-contracted (5.5 μ M) tracheas, the relaxant effects of 1 mM theophylline (THEO) or 0.24 mM enprofylline (ENPRO) were studied in the absence and presence of a sustained electrical stimulation (20 Hz for 12 min, pulse duration 0.5 ms, supramax. voltage; 0.1 μ M propranolol present). THEO and ENPRO were added when max. inhibition to nerve stimulation had developed (2-3 min). Mean $\pm$ SE results are shown.

	Tracheal tone before addition of xanthine mg	Relaxation by xanthines mg	%	n ^a
THEO alone	2600 $\pm$ 532	1150 $\pm$ 230	47.0 $\pm$ 4.2	4
THEO added during nerve stimulation	2563 $\pm$ 436	1200 $\pm$ 301	45.7 $\pm$ 5.8	4
ENPRO alone	2770 $\pm$ 537	848 $\pm$ 272	29.1 $\pm$ 7.6	5
ENPRO added during nerve stimulation	2950 $\pm$ 560	750 $\pm$ 215	25.1 $\pm$ 6.1	4
				NS

^a number of animals

^b Not significant ($p > 0.05$, Student's t-test)

reduced as has been earlier observed with xanthines and, more markedly, with β -adrenoceptor stimulants (Karlsson & Persson 1981).

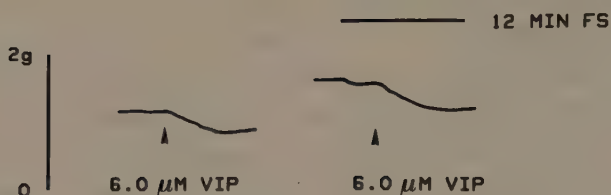


FIGURE 6

Original tracings showing that in a carbachol-contracted ($5.5 \mu\text{M}$) guinea-pig tracheal ring preparation already partially relaxed by a prolonged (12 min horizontal line above tracing) electrical stimulation (FS, see Fig. 1; $0.1 \mu\text{M}$ propranolol present), the relaxant effect of VIP is unchanged. In this preparation the relaxatory response to VIP is significantly larger than that produced by nerve stimulation.

Much work has focused on the non-adrenergic inhibitory nervous system in the airways and on the identity of the inhibitory neurotransmitter. Unfortunately, specific receptor antagonists are not available to the proposed neurotransmitters (except for adenosine) and, therefore, their possible involvement in the neurogenic response has to be studied indirectly. One possibility is to compare the non-adrenergic neurogenic control response with that obtained in the presence of a maximum concentration of a

putative transmitter substance. Thus, by occupying receptors available to the neurotransmitter it should be possible to reduce the magnitude or abolish the subsequent neurogenic response.

Based on findings with drugs blocking adenosine uptake Coleman & Levy (1974) suggested that adenosine may be the mediator of the non-adrenergic neurogenic relaxation. Although some results seem to disagree with a role for adenosine (Irvin et al 1982, Ito & Takeda 1982, Cameron et al 1983) this question is still open for discussion (Coleman 1980, Jones et al 1980, Souhrada et al 1980, Satchell 1982). Adenosine is an efficient relaxant of tracheas with a low tone (Coleman 1976, Jones et al 1980, Karlsson et al 1982) but, as shown in this study, adenosine was almost without effect in highly contracted tracheas. In contrast, the neurogenic response remained significant in these preparations. Further evidence against the involvement of adenosine in the neurogenic relaxation originates from the observation that theophylline, in adenosine-blocking concentrations (Persson et al 1982, Karlsson et al 1982), did not attenuate the non-adrenergic neurogenic inhibition (Table 1; see also Coleman 1980). In the gut, a purinergic nervous system is reported to mediate a non-adrenergic neurogenic relaxation (Burnstock 1981). A corresponding purinergic system with ATP as the neurotransmitter has been proposed to be present in the airways (Coleman & Levy 1974, Souhrada et al 1980). The relaxant actions of ATP in this study differed much from those of inhibitory field stimulation, particularly in preparations with a high tone (Fig 2). Moreover, indomethacin inhibited the ATP-induced relaxation but not the neurogenic inhibition suggesting that arachidonate cyclooxygenase products are involved in the ATP action (Kamikawa & Shimo 1976a, Jones et al 1980,

Lundblad et al 1984), and that ATP does not participate in the neurogenic inhibitory response of the guinea pig trachea. Adenine, the purine moiety of the nucleoside adenosine, is an efficient relaxant of airway smooth muscle with a mechanism of action different from that of adenosine (Coleman 1976, Farmer & Farrar 1976, Bach-Dieterle et al 1983). Consideration was given to adenine as a neurotransmitter candidate by Jones et al (1980). In agreement with previous work (Jones et al 1980, Bach-Dieterle et al 1983) we found that adenine-induced relaxations were not affected by drugs (theophylline, indomethacin) that interact with adenosine- or ATP-mediated effects. The relaxant characteristics of adenine were significantly different also from those of non-adrenergic inhibitory nerve stimulation, particularly in preparations with a high tone (Fig.2). In addition, neither adenosine nor adenine did interact in a significant way with the neurogenic relaxatory response. Taken together these results suggest that the purine derivatives may not be involved in the non-adrenergic, neural inhibition in guinea-pig trachea.

Matsuzaki et al (1980) proposed that VIP was the non-adrenergic, inhibitory neurotransmitter in the guinea-pig trachea. We observed that VIP-induced relaxation and neurogenic inhibition were similarly attenuated when the tracheal tone was raised by addition of carbachol (Fig. 2). This finding would by itself support the suggested role for VIP in non-adrenergic neurogenic inhibition. However, data on the interaction between VIP and nerve stimulation in highly contracted tracheas argue against this possibility. It was hypothesized that if VIP is released during nerve stimulation in concentrations large enough to affect the tone, supramaximal relaxant concentrations of exogenous VIP in the organ bath should abolish

or at least reduce the neural inhibitory response by occupation of receptors available to VIP. However, the presence of exogenous VIP did not affect the magnitude of the neurogenic inhibition. Nor was the relaxatory response to VIP affected by a prolonged, maximum nerve stimulation. The lack of interaction between relaxant effects of VIP and nerve stimulation does not support a role for VIP in the non-adrenergic inhibitory effects.

In conclusion, none of the examined transmitter candidates could be shown to be a likely participant in the non-adrenergic inhibitory effect. It cannot be excluded that the examined agents may, somehow, interact with each other and other transmitters to produce the neural inhibition. It is suggested that other neurotransmitter candidates for the non-adrenergic inhibitory nervous system than purines and VIP should be searched for.

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VI

Evidence against vasoactive intestinal polypeptide (VIP) as a dilator and in favour of substance P as a constrictor in airway neurogenic responses

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Propranolol-resistant neurogenic relaxation persisted in (carbachol-contracted) guinea-pig tracheae already relaxed by supramaximal concentrations of vasoactive intestinal polypeptide (VIP). Also, VIP relaxed preparations that were under neurogenic inhibition. In hilus bronchi, about 60% of a neurogenic contraction was atropine-resistant. (Arg⁵, D-Trp^{7,9}) SP_{5–11} specifically antagonized this contraction and those produced by exogenous substance P. Substance P, but not VIP, seems to be involved in nerve-mediated effects on guinea-pig airway tone.

Introduction Putative neurotransmitters for neural, atropine-resistant contraction and propranolol-resistant relaxation of guinea-pig airways have been proposed on the basis of immunohistochemical studies. Thus, substance P and vasoactive intestinal polypeptide (VIP) are present in nerve fibres in airways (Nilsson, Dahlberg, Brodin, Sundler & Strandberg, 1977; Uddman, Alumets, Desert, Håkanson & Sundler, 1978). Matsuzaki, Hamasaki & Said (1980) showed that the relaxant peptide, VIP, was released on nerve stimulation in guinea-pig trachea and that incubation of trachea with antibodies to VIP antagonized a neurogenic relaxation. However, the specificity for inactivation of endogenous VIP by the antibodies was not established. Furthermore, Matsuzaki *et al.* (1980) obtained surprisingly small mean relaxations (35 mg in tracheae with a tone of about 1000 mg) and diminutive responses (5 mg) were included in their calculations. We have examined responses to VIP, the effects of inhibitory nerve stimulation and the interaction between these two interventions in contracted guinea-pig tracheal preparations.

Grundström, Andersson & Wikberg (1981) found that nerve-induced contraction of guinea-pig hilus bronchi was entirely atropine-resistant. With antagonists to the contractile peptide, substance P, now available (Folkers; Hörig, Rosell & Björkroth, 1981), it was of interest to study the interaction between an antagonist and this neurogenic contraction. In isolated, electrically contracted hilus bronchi, the effect of a heptapeptide substance P antagonist (Bynke, Håkanson, Hörig & Leander, 1983) has been studied.

Methods Tracheal rings from guinea-pigs (body weight 300–500 g) (see Karlsson & Persson, 1981) were mounted in 2.5 ml organ baths containing Krebs solution (gassed with 95% O₂ and 5% CO₂) for recording of isometric tension changes (Grass FTO3, Grass polygraph model 5). Tubal segments were taken from the hilus bronchi of the left and right caudal lobes and mounted as described above (initial tension 0.3 g). Tracheal and bronchial preparations were electrically stimulated (Grass S88 stimulator) using field stimulation via platinum electrodes. A supramaximal voltage was used which produced responses that were blocked by tetrodotoxin (1 µg/ml). When studying inhibitory nerve-stimulation in tracheae, propranolol (0.1 µM) was always present. Mean ± s.e. mean values from preparations obtained from different animals are given.

Drugs used were: atropine sulphate (Sigma), carbachol chloride (Sigma), propranolol dihydrochloride (Hässel), (Arg⁵, D-Trp^{7,9}) substance P_{5–11} (gift from Dr Hörig, Ferring), tetrodotoxin (Sankyo) and vasoactive intestinal polypeptide (VIP, V. Mutt.). All drugs were dissolved in 0.85% NaCl solution.

Results

Relaxation and vasoactive intestinal polypeptide

Tracheal preparations developed a spontaneous tone of 1780 ± 233 mg (*n* = 9). Maximum relaxation induced by a train of stimuli for 2 min (pulse duration 0.5 ms and frequency 20 Hz) reduced tracheal tone by 1314 ± 130 mg (in the presence of 0.1 µM propranolol). This relaxation is considerably larger than that reported by Matsuzaki *et al.* (1980).

Maximum effective concentrations of VIP (23.0 µM) reduced the tone in carbachol-contracted tracheal preparations by 29.8 ± 4.1% (*n* = 4). A comparable maximal relaxation, 24.5 ± 2.9% (*n* = 8), was produced by nerve stimulation, using the same stimulus parameters as above. Carbachol 5.5 × 10⁻⁶ M was used. This concentration raised the tone to 2620 ± 200 mg (*n* = 12) and corresponded to carbachol's EC₉₅. In carbachol-contracted tracheal preparations with a supramaximal concentration of

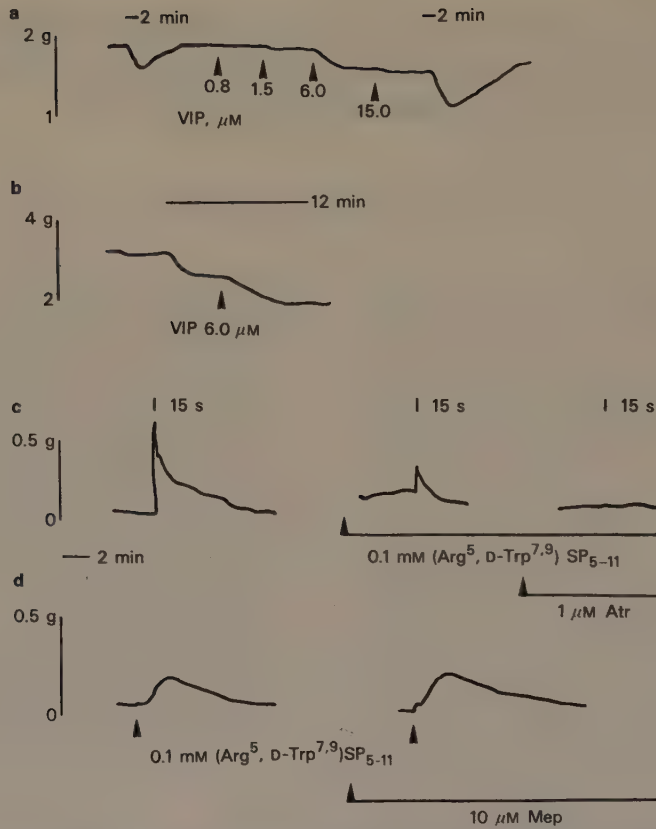


Figure 1 (a) In a carbachol-contracted ($5.5 \mu\text{M}$) tracheal ring in the presence of propranolol ($0.1 \mu\text{M}$), electrical field stimulation (horizontal lines above the tracing) produces a relaxant effect that is not changed by the presence of maximally effective concentrations of vasoactive intestinal polypeptide (VIP). (b) In a carbachol-contracted ($5.5 \mu\text{M}$) tracheal ring preparation already relaxed by prolonged electrical stimulation (horizontal line), VIP is still an efficient relaxant ($0.1 \mu\text{M}$ propranolol present). (c) In a tubal segment of hilus bronchi electrical stimulation (vertical lines) produces contraction, the major part of which is antagonized by a heptapeptide substance P antagonist. The minor part is antagonized by atropine (Atr). Time calibration (2 min) is shown to the left of the figure. (d) A transient contraction is produced by the substance P antagonist in a bronchial preparation. The contraction is not attenuated by mepyramine (Mep). Time calibration as in (c).

VIP present (VIP was added until no further relaxation was recorded and then its concentration was doubled) the inhibitory response to nerve stimulation was intact (Figure 1a). Moreover, the mean relaxation induced by $6.0 \mu\text{M}$ VIP ($19.9 \pm 6.5\%$, $n = 5$; corresponding to the EC_{70}) was unchanged ($18.7 \pm 3.2\%$, $n = 5$) when evaluated during a maximal inhibitory response to prolonged nerve stimulation (Figure 1b).

Contraction and substance P Electrical stimulation using a train of stimuli for 15 s (pulse duration 0.6 ms and frequency 15 Hz) of the hilus bronchi produced reproducible contractile responses. Atropine, $1 \mu\text{M}$, reduced these by $37.7 \pm 4.9\%$ ($n = 9$). Atropine-resistant contractions had a mean tension of $201 \pm 36 \text{ mg}$ ($n = 13$).

Treatment of bronchial preparations with (Arg^5 , $\text{D-Trp}^{7,9}$) SP_{5-11} for 15 min reduced the magnitude of

the electrically induced contraction, the residual response being completely blocked by atropine (Figure 1c). In the presence of atropine ($1 \mu\text{M}$) (Arg^5 , D-Trp 7,9) SP_{5-11} 10^{-5} M , $3 \times 10^{-5} \text{ M}$ and 10^{-4} M inhibited the amplitude by $16.2 \pm 6.9\%$ ($n = 5$), $63.5 \pm 8.2\%$ ($n = 5$) and $77.1 \pm 3.4\%$ ($n = 8$), respectively.

(Arg^5 , D-Trp 7,9) SP_{5-11} (10^{-5} M to 10^{-4} M) produced a transient ($< 15 \text{ min}$) contractile response in 16 of 36 preparations. Treatment with mepyramine ($3 - 10 \mu\text{M}$) did not affect this contraction (Figure 1d).

The concentration-response relationship to histamine, carbachol, and substance P was examined in the absence and presence of 10^{-4} M (Arg^5 , D-Trp 7,9) SP_{5-11} . Only substance P-induced contractions were antagonized. Its concentration-response line was shifted to the right 2.3 times ($P < 0.05$, two-tailed Student's *t* test). The concentration-response lines to histamine and carbachol tended to be shifted to the left although this was not statistically significant ($P > 0.05$). The following EC_{50} values were obtained; histamine ($n = 3$) $14.6 \pm 4.8 \mu\text{M}$ and $8.2 \pm 0.8 \mu\text{M}$, carbachol ($n = 4$) $1.65 \pm 0.61 \mu\text{M}$ and $0.83 \pm 0.19 \mu\text{M}$, substance P ($n = 3$) $26.3 \pm 6.4 \mu\text{M}$ and $62.7 \pm 20.2 \mu\text{M}$ in the absence and presence, respectively, of the antagonist.

Discussion In the presence of maximally effective concentrations of VIP the neurogenic inhibition of carbachol-contracted tracheae persisted, suggesting that the relaxation induced by nerve stimulation is unrelated to VIP. Against this interpretation it could perhaps be argued that a neurotransmitter, when added exogenously, may not reach subsynaptic receptors because of diffusion barriers and thus be less

effective than stimulation of nerves. This argument may not be valid, however, because the effect of VIP was unchanged when the peptide was added during prolonged electrical stimulation, i.e. when subsynaptic receptors might be expected to be fully activated.

In preparations of hilus bronchi about 60% of the magnitude of the neurogenic contractions was atropine-resistant. This finding suggests that both non-cholinergic (Grundström *et al.*, 1981) and cholinergic neural contractions can be induced in these bronchi. The non-cholinergic contraction was antagonized by (Arg^5 , D-Trp 7,9) SP_{5-11} in a concentration-dependent way. This peptide transiently contracted some preparations via a mechanism apparently unrelated to histamine release since it was mepyramine-resistant. Some specific antagonism by (Arg^5 , D-Trp 7,9) SP_{5-11} of contractions induced by exogenous substance P was also shown. The data thus support a mediator role for substance P as well as acetylcholine in neurogenic contraction of the hilus bronchi.

During the preparation of this communication we have become aware of studies from two other laboratories. Håkanson and Leander (personal communication) observed that a substance P antagonist inhibited atropine-resistant neurogenic contractions in guinea-pig stem bronchi, while Lundberg, Saria, Brodin, Rosell & Folkers (1983) showed that in the presence of mepyramine, an undecapeptide substance P antagonist completely antagonized neurogenic contractions of guinea-pig hilus bronchi.

Thus, this study of guinea-pig airways provides evidence against a role for VIP as a neurogenic dilator of the trachea, but supports a role for substance P and acetylcholine as neurally released constrictors of hilus bronchi.

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VII

Local anesthetics selectively inhibit non-cholinergic neural contractions in guinea-pig airways

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Electrical field stimulation of guinea-pig isolated trachea and bronchus may produce two types of contractions (Grundström et al. 1981) which can be classified as cholinergic, atropine-sensitive and non-cholinergic, substance P-antagonist-sensitive (Karlsson & Persson 1983, Lundberg et al. 1983). Supporting their neural origin these actions are prevented by tetrodotoxin. Conceivably, local anesthetic agents would also be effective but little is known quantitatively about the interaction between local anesthetics and airway neurogenic contractions.

Local anesthetic drugs, and in particular inhaled lidocaine, are being employed to evaluate the role of nervous mechanisms in respiratory disorders such as bronchial asthma (e.g. Griffin et al. 1982, Tullet et al. 1982, Sheppard et al. 1983) and these agents may be useful in the treatment of hyperreactive airways. It was thus of interest to examine the effects of selected local anesthetics on neural contractions of airway smooth muscle. In the present study inhibitory actions of lidocaine and prilocaine were evaluated in electrical field stimulated hilus bronchi and tracheas, obtained from guinea-pigs.

Guinea-pigs of both sexes (bwt 300–500 g) were killed by a blow to the head. The lung with the adjoining trachea was rapidly excised and placed in Krebs solution. Open tracheal rings and tubal segments of hilus bronchi were prepared and mounted on metal prongs in organ baths containing 2.5 ml of heated (37°C), carbogenized Krebs solution (see Karlsson & Persson 1981, 1983). Initial tension was set to 0.6 g and 0.3 g in tracheal and bronchial preparations, respectively. Isometric tension changes were recorded (Grass FTO3).

Tracheal and bronchial preparations were electrically stimulated (field stimulation with a Grass S11 stimulator) with a pulse duration of 0.3 ms at a maximal voltage producing responses that were completely blocked by 3 µM tetrodotoxin. The responses were reproducible, permitting the study of

several concentrations of a drug within each preparation.

Mean±SE IC_{50} values are given. (The IC_{50} value is defined as the concentration of drug producing 50% inhibition of the neural response.) Slopes of concentration-response relationships were calculated by use of linear regression analysis. Statistical calculations were made by use of the Student's *t*-test for unpaired observations.

Drugs used were atropine sulphate (Sigma), indomethacin (Sigma), lidocaine hydrochloride (Astra), D-(-)-prilocaine hydrochloride (Astra), and propranolol dihydrochloride (Hässel). Indomethacin was dissolved in 5% NaHCO₃ and diluted in Krebs solution. The other drugs were both dissolved and diluted in Krebs solution.

Tracheal rings spontaneously gained a tone which was almost completely abolished by the addition of 3 µM indomethacin. In the presence of this concentration of indomethacin and of 0.1 µM propranolol, electrical nerve stimulation of tracheas with trains of pulses (10 Hz for 4 s, 0.03 trains per s) produced submaximal transient contractions that were completely inhibited by 1 µM atropine. The magnitude of the contractions were between 30 mg and 240 mg in different preparations. Both lidocaine and prilocaine inhibited these in a concentration-related way (Fig. 1). Stable responses were obtained at each concentration level. This allowed evaluation of the effects of cumulatively increasing concentrations of the drugs. Lidocaine was somewhat more potent than prilocaine, IC_{50} values being $0.9 \pm 0.2 \times 10^{-4}$ M (*n*=6) and $2.2 \pm 0.5 \times 10^{-4}$ M (*n*=4), respectively.

Electrical nerve stimulation (15 Hz for 15 s) contracted tubal segments of the hilus bronchi. The contractions were only partly blocked by atropine (see Karlsson & Persson 1983). In the presence of 1

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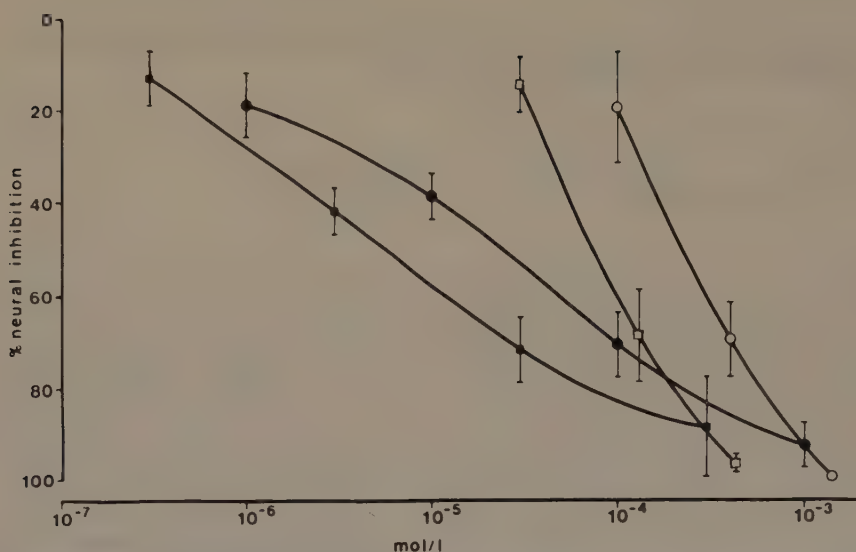


Fig. 1. Lidocaine (squares) and prilocaine (circles) concentration dependently inhibited both non-cholinergic contractions (filled symbols) and cholinergic contractions (open symbols) in electrical field stimulated bronchi and tracheas obtained from guinea-pigs. Lidocaine was slightly more potent than prilocaine. The non-cholinergic response was inhibited more readily than the cholinergic contraction, although the slope of the concentration-response line for the former effect was less steep. Vertical bars represent SE. $n=4-6$.

μM atropine, both lidocaine and prilocaine given as single doses 5 min before start of stimulation inhibited the contractions in a concentration-dependent manner (Fig. 1). IC_{50} values for lidocaine and prilocaine were $1.3 \pm 0.6 \times 10^{-5}$ M ($n=4$) and $2.6 \pm 0.8 \times 10^{-5}$ M ($n=4$). The slopes of concentration-response curves to the local anaesthetics in the hilus bronchi were less steep than those obtained in the tracheal preparations ($p < 0.001$, Fig. 1).

Both non-cholinergic and cholinergic contractions were thus blocked by the local anesthetic agents. Lidocaine was 2 to 3 times more potent than prilocaine which is in agreement with the anesthetic potency ratio between these two agents determined in the isolated frog sciatic nerve preparation (Åström & Persson 1961). However, lidocaine and prilocaine were both 10 times more potent (at the level of IC_{50}) as inhibitors of non-cholinergic than cholinergic contractions. This finding suggests that the non-cholinergic contraction involve stimulation of sensory non-myelinated nerve fibres (C-fibres) since these may be particularly sensitive to interruption by local anaesthetics.

The present data also suggest that local anesthetic agents may be used experimentally to specifically inhibit non-cholinergic, possibly peptidergic, neural contractions of airways smooth muscle.

Based on immunochemical localisation and analysis of substance P and on a number of experiments with capsaicin that depletes sensory neurones of i.a. substance P, this peptide has been suggested to mediate the non-cholinergic bronchoconstriction in the guinea-pig (see Lundberg et al. 1983). Apparently this suggestion was substantiated by results obtained with the substance P-antagonists (D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹), SP and (Arg⁵, D-Trp^{7,9}) SP₅₋₁₁. Like lidocaine and prilocaine in the present study, the substance P-antagonists inhibited the non-cholinergic contraction without antagonising the cholinergic response (Karlsson & Persson 1983, Lundberg et al. 1983). However, is it not known whether these substance P-antagonists also have local anaesthetic properties.

In conclusion, the present study of guinea-pig airway smooth muscle preparations has shown that the local anesthetic agents lidocaine and prilocaine

inhibit the main part of a non-cholinergic neural contraction before they reach concentrations at which the cholinergic response is attenuated.

We thank Dr Claes Post for supplying the local anesthetic agents.

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VIII

SUBSTANCE P ANTAGONISTS AND THE ROLE OF TACHYKININS IN NON-CHOLINERGIC BRONCHOCONSTRICTION

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Summary

Electrical field stimulation of guinea-pig isolated hilus bronchi induced tetrodotoxin-sensitive contractions of which only a minor part could be inhibited by atropine. The remaining non-cholinergic bronchoconstriction was antagonized by a heptapeptide and an undecapeptide substance P (SP) analogue (Arg⁵,D-Trp^{7,9})SP₅₋₁₁, IC₅₀ = 24.0 μ M and (D-Pro²,D-Trp^{7,9})SP, IC₅₀ = 10.0 μ M. Of the exogenously added tachykinins, both eledoisin (8 times) and physalaemin (3 times) were more potent bronchoconstrictors than SP. Pretreatment with the SP-analogues shifted concentration-response curves to the tachykinins to the right, eledoisin being most readily antagonized. (Arg⁵,D-Trp^{7,9})SP₅₋₁₁ also antagonized the neural response more readily than that of SP. In addition, in the frog isolated sciatic nerve preparation the two SP-analogues were found to possess potent lidocaine-like neurodepressant actions which further complicated the interpretation of the neural inhibitory effects of these compounds. It is concluded that if a tachykinin contributes to non-cholinergic bronchoconstriction, an eledoisin-like peptide is a more likely candidate than SP itself. Since SP-antagonists may have local anaesthetic properties their value as tools in neurophysiology seems limited. Inferentially, the non-cholinergic bronchoconstrictive neurotransmitter remains to be identified.

Introduction

The undecapeptide substance P (SP) has been given consideration as a possible transmitter of non-cholinergic neurogenic responses in the lung. Histochemical studies have demonstrated nerves with immunoreactivity to this particular tachykinin in guinea-pig and humanhuman airways (1,2,3). SP is further known to be a bronchoconstrictor (e.g. 4) and may produce pro-inflammatory actions in

the airways (see 5,6). Electrical field-stimulation of guinea-pig bronchi produces a neural contraction that is resistant to atropine (7) but is inhibited by SP-analogues having SP-antagonistic properties (5,8). If the SP-analogues are truly specific antagonists such observations would indeed constitute evidence for SP being the mediator of the non-cholinergic contraction.

SP-antagonists are widely used in the evaluation of possible roles of SP in physiology and pathophysiology (see 9,10). We have now examined effects of a heptapeptide, (Arg⁵,D-Trp^{7,9})SP₅₋₁₁ and an undecapeptide, (D-Pro²,D-Trp^{7,9})SP analogue in guinea-pig isolated hilus bronchi. Both peptides have been shown to possess SP-antagonistic properties (11,12), but their ability to antagonise effects of other tachykinins in airways is not known. It is also not known if an effect unrelated to tachykinin antagonism, e.g. local anaesthesia, is involved when these analogues inhibit neural responses. These aspects on the specificity of SP antagonists are dealt with in the present study.

Methods

Bronchial preparation

Tubal segments were dissected from guinea-pig (Dunkin-Hartley, body weight 300-600 g) hilus bronchi of the left and right caudal lobes. The bronchi were mounted in organ baths containing 2.5 ml of heated (37°C) Krebs solution (composition in mM: NaCl 118.0, KCl 4.6, CaCl₂ 2.5, MgSO₄ 1.15, NaHCO₃ 24.9, KH₂PO₄ 1.15, glucose 5.5; pH=7.4) gassed with 5% CO₂ in oxygen. Tension changes in the bronchi were measured isometrically with Grass strain-gauge transducers (FT03) and recorded on a Grass polygraph model 7. The preparations were given an initial tension of 0.3 to 0.4 g and were left to stabilize for about an hour during which time repeated washings were performed. Drugs were added by injection to the organ bath either as a single dose or in a cumulative progression of concentrations until no further effect was obtained with the next higher concentration. In some experiments bronchial preparations were electrically stimulated (Grass S88 stimulator) using field stimulation. A maximum voltage was used.

Sciatic nerve preparation

Both sciatic nerves were isolated from decapitated *Rana pipiens* and immediately put into cold (4°C) MOPS buffer (composition in mM: NaCl 115.0, CaCl₂ 2.0, KCl 2.5, 3-(N-morpholino)propanesulfonic acid 5.0, glucose 5.6; pH adjusted to 7.25 with NaOH). The nerves were desheathed, split in halves longitudinally and mounted in a sucrose gap recording chamber (13) at room temperature (20°C). The gap of the nerve was perfused with 0.18 M sucrose in distilled water and the other parts of the nerve kept in MOPS buffer. The nerves were continuously electrically stimulated (Grass S48 stimulator) via Ag/AgCl electrodes at supramaximal voltage with a frequency of 1 Hz and a pulse duration of 0.05 ms. The compound action potential (APc) was obtained with recording electrodes and amplified in a Neurolog model NL 104AC preamplifier and via a Neurolog model NL 125 filter recorded on a Tektronix model 468 digital storage oscilloscope, which was on line to a Hewlett-Packard HP86 computer

where the graphs were stored. Drugs were added by injection to one chamber of the organ bath.

Studies with SP-analogues

In studies evaluating possible antagonistic properties of SP-analogues in bronchial preparations, electrical nerve stimulation or concentration-response (C/R) relationships to the tachykinins were obtained in the presence of (D-Pro²,D-Trp^{7,9})SP or (Arg⁵,D-Trp^{7,9})SP₅₋₁₁. (D-Pro²,D-Trp^{7,9})SP, $\geq 3\mu\text{M}$, transiently (10-30 min) contracted the bronchi. The contraction produced by $10\mu\text{M}$ of the antagonist was resistant to atropine ($1\mu\text{M}$) but reduced by $36.7\pm 9.8\%$ by mepyramine ($1\mu\text{M}$, n=4). The heptapeptide (Arg⁵,D-Trp^{7,9})SP₅₋₁₁, $\geq 10\mu\text{M}$, transiently (10-20 min) contracted 17 of 36 bronchi. Apparently this contraction was unaffected by atropine ($1\mu\text{M}$) and mepyramine ($3\text{--}10\mu\text{M}$) (see 8). Evaluation of C/R curves or electrical nerve stimulation was not begun until the SP-analogue induced contractions had faded completely. In the sciatic nerve preparation, SP-analogues were added during continuous electrical stimulation.

Statistical procedures

Geometric mean $\pm$ SEM values were calculated for each concentration of the drugs. pD₂ values are defined as the negative log molar concentration of a drug producing 50% of carbachol's maximal response and are geometric mean $\pm$ SEM results from each individual C/R curve. The pIC₅₀ value is defined as the negative log molar concentration of a drug reducing the neural response or the APC by 50%. Geometric mean $\pm$ SEM values are also obtained from individual curves. In the Results section mean $\pm$ SEM values are shown. Statistical differences were analysed by use of the Student's t-test for unpaired observations.

Substances

Drugs used were atropine sulphate (Sigma), mepyramine maleate (May & Baker), eledoisin, physalaemin and substance P (SP) (BACHEM or Peninsula), (D-Pro²,D-Trp^{7,9})SP (Peninsula or Ferring, Kiel), (Arg⁵,D-Trp^{7,9})SP₅₋₁₁ (Ferring, Kiel) and tetrodotoxin (TTX, Sankyo). All drugs were both dissolved in and diluted with the proper buffer solution immediately before the experiments were begun and kept on ice throughout them. Before addition of a drug solution to the sciatic nerve, its pH was adjusted to 7.25.

Results

Effects of SP-analogues on non-cholinergic neural contractions of bronchi

Electrical field stimulation using a train of stimuli for 15 s (pulse duration 0.3 ms and frequency 15 Hz) of guinea-pig isolated hilus bronchi produced a contraction consisting of a cholinergic and a non-cholinergic (atropine-resistant) component (see 8). The contraction was blocked by TTX ($3\mu\text{M}$) confirming its neural origin. In the presence of atropine ($1\mu\text{M}$), electrically induced contractions were reproducible when repeated 4 to 5

times, allowing the examination of several single concentrations of an SP-analogue in each preparation. Both (D-Pro²,D-Trp^{7,9})SP and (Arg⁵,D-Trp^{7,9})SP₅₋₁₁ inhibited the non-cholinergic neural contraction in a concentration-related way (Fig 1).

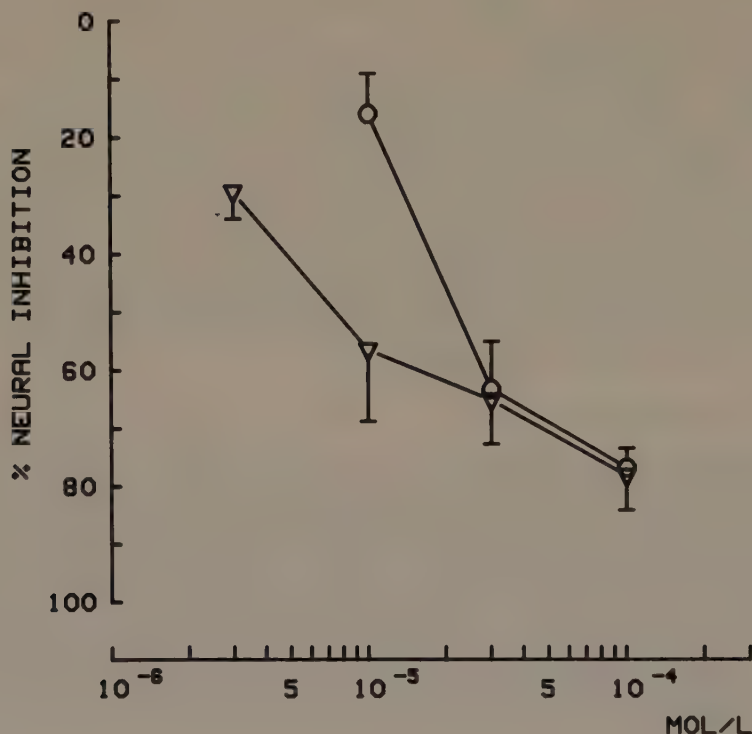


FIG. 1

Inhibition of the non-cholinergic neural contraction of guinea-pig hilus bronchi by two substance P (SP)-analogues. Electrical field stimulation of atropinized (1 μ M) bronchi produced contractions, reduced in the presence of either (D-Pro²,D-Trp^{7,9})SP (∇ , n=5) or (Arg⁵,D-Trp^{7,9})SP₅₋₁₁ (O, n=5). Mean curves are shown and vertical lines indicate SEM.

An average maximum inhibition by about 80% was achieved with each SP-analogue at 100 μ M. Mean pIC₅₀-values were 4.98 ± 0.14 (n=8) for (D-Pro²,D-Trp^{7,9})SP and 4.62 ± 0.08 (n=5) for (Arg⁵,D-Trp^{7,9})SP₅₋₁₁.

Effects of SP-analogues on tachykinin-induced bronchial contractions

Isolated hilus bronchi were concentration-dependently contracted by the tachykinins used in this study. Bronchial sensitivity to the tachykinins was unaffected by repeated exposure since reproducible C/R relationships could be obtained at least 2 to 3 times in individual preparations. Maximum concentrations used (100 μ M) of SP, physalaemin or eledoisin produced contractions that

amounted to $85.8 \pm 1.7\%$ ($n=6$), $84.6 \pm 3.0\%$ ($n=8$) and $91.9 \pm 2.1\%$ ($n=7$), respectively, of carbachol's maximum (Fig 2). The maximum

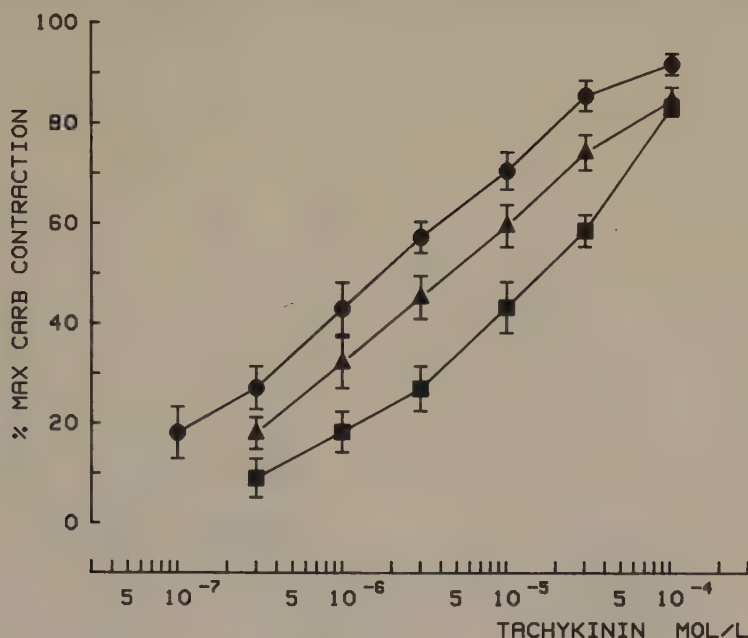


FIG. 2

Contractile concentration-response relationships to substance P (■, $n=9$), physalaemin (▲, $n=9$) and elledoisin (●, $n=7$) in guinea-pig isolated hilus bronchi. Peptides were added cumulatively to the bronchi and the response obtained is expressed as per cent of carbachol's maximum (100 μ M, added on top of each curve). Mean curves are shown and vertical lines indicate SEM.

degrees of contraction did not differ significantly ($P>0.05$) between the tachykinins. Elledoisin was most potent, being approximately 8 times and 3 times more potent than SP and physalaemin (Table 1). In the presence of 100 μ M of either (D-Pro², D-Trp^{7,9})SP or (Arg⁵, D-Trp^{7,9})SP₅₋₁₁ the C/R curves to the tachykinins were shifted rightwards. This is illustrated in Fig 3 for SP-induced contractions. The curve appeared to be shifted in parallel, but the full curve could not be obtained in the presence of 100 μ M of antagonist (see Fig 3). Since in the presence of the SP-analogues, the effects of tachykinins at maximum used concentrations (100 μ M) were depressed compared with carbachol, pD_2 values were calculated relative to carbachol's maximum. SP-analogues antagonized bronchial contractions by elledoisin more than contractions by the other tachykinins (Table 1).

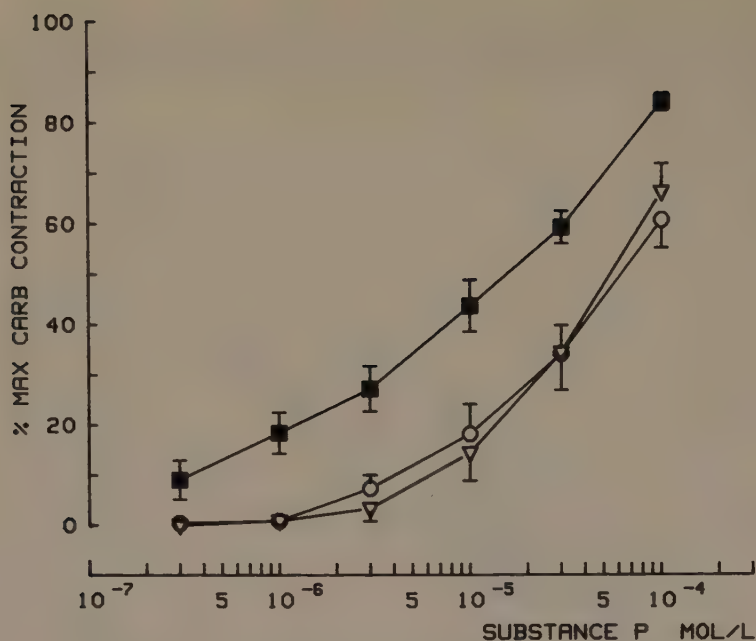


FIG. 3

Cumulative concentration-response curves to exogenous substance P (SP; ■, n=9) obtained in guinea pig hilus bronchi in the absence and presence of two SP-analogues. Pretreatment with 100 μ M of either (Arg⁵,D-Trp^{7,9})SP₅₋₁₁ (○, n=5) or (D-Pro²,D-Trp^{7,9})SP (▽, n=5) shifted the SP curve rightwards 4.6 and 4.0 times (at the level of EC₅₀), respectively. Mean results are shown and vertical lines indicate SEM.

TABLE I

(D-Pro²,D-Trp^{7,9})SP (Arg⁵,D-Trp^{7,9})SP₅₋₁₁

Substance P	pD ₂	4.87±0.11	4.27±0.13**	4.21±0.12**
	n	9	5	5
	ratio		4.0	4.6
Physala-	pD ₂	5.38±0.14	4.73±0.20*	4.62±0.13**
emin	n	9	5	5
	ratio		4.5	5.8
Eledoisin	pD ₂	5.79±0.12	4.50±0.15***	4.43±0.07***
	n	7	3	4
	ratio		19.5	22.9

Mean pD₂ (±SEM) values to various tachykinins obtained in guinea-pig isolated hilus bronchi in the absence and presence of two substance P (SP)-antagonists (100 μ M). Cumulative concentration-response relationships were constructed to the tachykinins. The pD₂ value is defined as the negative log molar concentration of tachykinin producing 50% of a maximum carbachol (100 μ M) contraction. * denotes level of significance (compared to the pD₂ value obtained without antagonist; *=P<0.05, **=P<0.01, ***=P<0.001, Student's t-test).

In separate experiments, the effects of (Arg⁵,D-Trp^{7,9})SP₅₋₁₁ (100 μ M) on SP-induced (10 μ M) and non-cholinergic nerve-induced contractions were compared. The amplitude of the induced contractions was similar, but the SP-analogue inhibited neural contractions significantly ($P<0.05$) more than SP-induced contractions. (Fig 4).

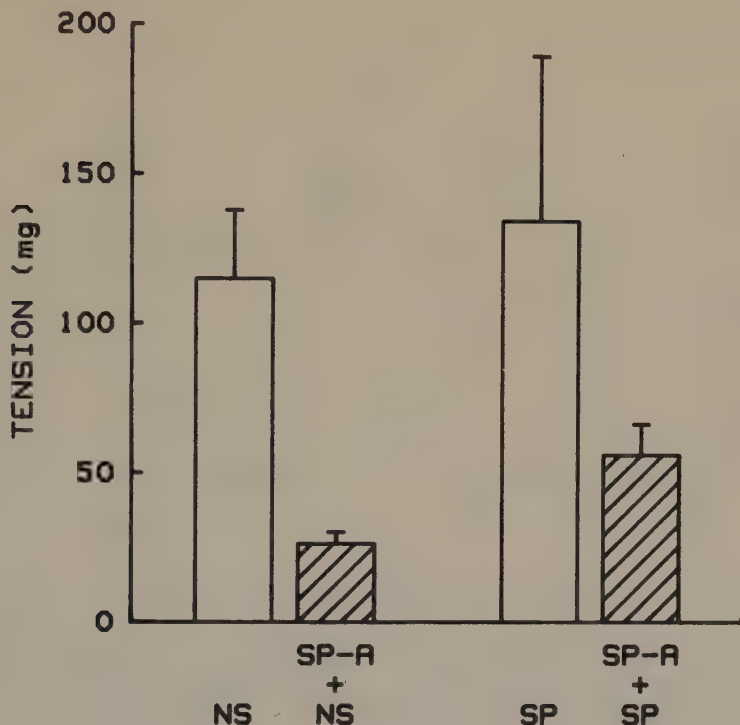


FIG. 4

Effect of (Arg⁵,D-Trp^{7,9})SP₅₋₁₁ (SP-A, 100 μ M) on contractions of the guinea-pig hilus bronchi produced by non-cholinergic nerve stimulation (NS, see Methods) or by exogenous substance P (SP, 10 μ M). The SP-A inhibited significantly the neural contraction ($P<0.01$). The histogram illustrates that, despite a similar magnitude of the average control contractions, SP-A inhibited NS-induced contractions significantly more than SP-induced contractions ($P<0.05$). Open bars represent mean tension induced by NS ($n=8$) or SP ($n=7$) in the absence, and hatched bars mean tension (NS, $n=8$; SP, $n=7$) in the presence of SP-A. Vertical lines illustrate SEM.

Effects of SP-analogues on APC in the sciatic nerve

Continuous electrical stimulation with a frequency of 1 Hz (pulse duration 0.05 ms) of frog isolated sciatic nerves produced APC of a constant amplitude. Addition of increasing concentrations of (D-Pro²,D-Trp^{7,9})SP or (Arg⁵,D-Trp^{7,9})SP₅₋₁₁ to the nerve bath gradually diminished the amplitude of the APC (Fig 5). Buffer alone had no effect on the APC ($n=8$). Complete blockade was obtained with 400 μ M of either SP-analogue (Fig 5).

50% inhibition of the APC was produced by 155 μ M and 130 μ M, respectively, of (D-Pro²,D-Trp^{7,9})SP and (Arg⁵,D-Trp^{7,9})SP₅₋₁₁.

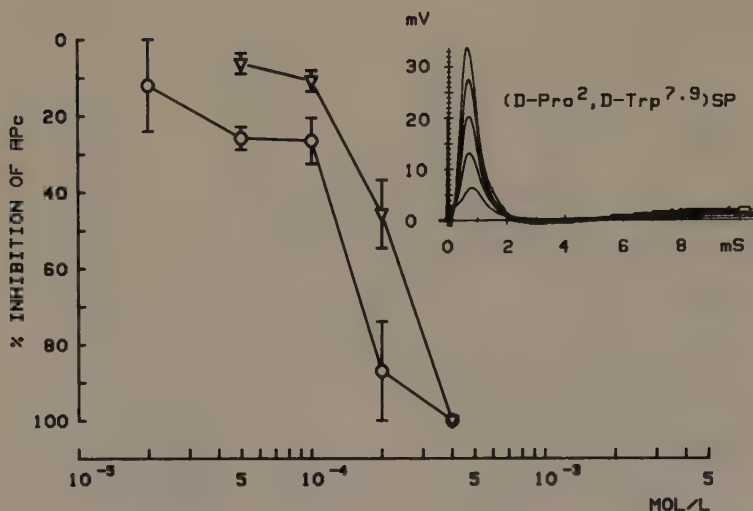


FIG. 5

Inhibition of the compound action potential (APC) in electrically stimulated (see Methods) frog isolated sciatic nerves by two substance P (SP)-analogues. Single concentrations of (D-Pro²,D-Trp^{7,9})SP (▽, n=3-4) or (Arg⁵,D-Trp^{7,9})SP₅₋₁₁ (○, n=3-4) were added to continuously stimulated nerve preparations. Mean curves are shown and vertical lines indicate SEM. Insert: original tracing showing the inhibitory effect of 200 μ M of (D-Pro²,D-Trp^{7,9})SP. Top curve represents the APC before addition of SP-analogue and below are APCs obtained 5, 10, 15 and 20 min after addition.

Since commercially available preparations of SP-analogues may contain variable degrees of acetate ions, acetic acid (12 mM) was added to the nerve preparation. However, it did not affect the amplitude of the APC (n=3) indicating that the inhibition of neural conduction was not due to this possible contaminant.

Discussion

Isolated guinea pig hilus bronchi were contracted by exogenously added tachykinins. These peptides produced strong contractions of a degree similar to that of carbachol. Of the tachykinins studied, eledoisin was found to be significantly more potent than SP and physalaemin. These limited data would suggest that the SP-E type of receptors (see 14,15) are present in guinea-pig bronchi. It is of interest that although non-cholinergic neural contractions cannot be evoked in tracheal preparations the tachykinins con-

tracted the tracheal muscles and exhibited an order of potency (1,16) similar to that found in hilus bronchi where the neural non-cholinergic contractions are pronounced. Furthermore, each tachykinin was between one and two orders of magnitude less potent in the hilus bronchi (this study) than in the trachea (16, unpublished observations by Finney and Karlsson). Hence, the relevance of observations made in the trachea may be in doubt for two reasons: a non-cholinergic neural contraction cannot be obtained in this preparation, and the potency of the tachykinins is markedly different from that in the hilus bronchi.

Fragments of different lengths of SP have been synthesized with the ability to antagonize SP-induced actions, (but not those produced by other types of mediators), in guinea-pig isolated smooth muscle preparations such as the ileum (11), the taenia coli (17) and the trachea (16). The present study was conducted with an undecapeptide ($\text{D-Pro}^2, \text{D-Trp}^{7,9}$)SP and a heptapeptide ($\text{Arg}^5, \text{D-Trp}^{7,9}$)SP₅₋₁₁ analogue. These compounds illustrate a chemical variation among SP antagonists and may be representative for other analogues as well. SP-analogues may produce effects of their own. For example, they may contract smooth muscle (e.g. 11) and they may release histamine from various cell preparations (e.g. 18). Both selected SP-antagonists produced transient contractions of the isolated bronchi but only ($\text{D-Pro}^2, \text{D-Trp}^{7,9}$)SP seemed to act in part via the release of endogenous histamine (see 8,19).

In agreement with two earlier communications (8,19) both SP-analogues were able to suppress the non-cholinergic neural contractions of isolated bronchi. The compounds were not very potent as inhibitors. 30-100 μM concentrations had to be used to obtain a substantial (60-70%) reduction of the neural response. Still, as shown by the actions of one analogue they appeared more potent to inhibit neural responses than to antagonize the bronchial contractions produced by exogenously added SP (see Fig 4). This finding may be related to the observation that the SP-analogues could distinguish also between different tachykinins. The effects of eledoisin were most readily inhibited. The rightward shift of its C/R curve was about four times larger than that of the SP or physalaemin C/R curves. During the preparation of this report, Mizrahi et al (16) published a similar preferential action against eledoisin-induced contractions by undecapeptide SP-analogues in the guinea-pig trachea.

Thus, interactions between SP-antagonists and neural or tachykinin-induced responses may suggest that the non-cholinergic contraction of hilus bronchi is mediated by another tachykinin than SP itself. This possibility has support in some recent findings. The presence of eledoisin and physalaemin has now been demonstrated in nerve fibres within the airways (20, J.M. Lundberg, personal communication). Also, the elucidation of the structure of two mammalian SP-precursor molecules showed that one of them contained a sequence (named substance K) closely related to the amphibian tachykinin kassinin (21). This sequence seems to have biological actions similar to those of kassinin (22,23) and eledoisin (see 14,24). Inferentially, these observations together

with the present data may suggest that an eledoisin-like tachykinin is involved in the non-cholinergic bronchial contraction.

However, if SP-antagonists have unspecific neurodepressant actions it may be doubtful if tachykinins are at all involved in the non-cholinergic neural contraction. The possibility of unspecific depressant actions was examined in the frog isolated nerve that is a commonly employed preparation in the evaluation of local anaesthetic agents. Both (D-Pro²,D-Trp^{7,9})SP and (Arg⁵,D-Trp^{7,9})SP₅₋₁₁ completely blocked conduction in the sciatic nerve (Fig. 5). They have further been found to be about 4 times more potent than lidocaine whereas SP itself does not block conduction (Post, Karlsson, Butterworth, Persson & Strichartz, in preparation). Lidocaine has earlier been shown to inhibit the non-cholinergic bronchoconstriction with an EC₅₀ value of about 13 μ M (25) thereby being about equipotent with the SP-antagonists. Both types of agents were actually more potent to inhibit noncholinergic bronchoconstriction than to block conduction in the sciatic nerve preparation (about 65 times larger concentrations of lidocaine but only 10 times (or less) larger concentrations of SP-antagonists were needed). It is thus quite possible that a local anaesthetic-like action of SP-analogues contributed to the inhibition of the non-cholinergic neural contraction in hilus bronchi.

An alternative to employing SP-antagonists in the elucidation of the non-cholinergic neurotransmitter would be to make the bronchi tachyphylactic to the tachykinins and then examine neural effects. However, no sign of tachyphylaxis could be found with these peptides in the bronchi, contrary to what has been shown for example in guinea-pig ileum (14) and, therefore, this method could not be used to examine the possible role for a tachykinin in the neural response.

In conclusion, two structurally different SP-analogues inhibited non-cholinergic neural contractions and those mediated by three exogenously added tachykinins in the guinea-pig hilus bronchi. Both the neural and the eledoisin responses were inhibited more readily than that of SP. In addition, the SP-analogues were found to possess lidocaine-like neurodepressant actions which further complicate the interpretation of data obtained with these compounds. Inferentially, the identity of the non-cholinergic neurotransmitter in the airways remains to be determined.

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